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(54) Title: METHODS AND REAGENTS FOR DETECTING FUNGAL PATHOGENS IN A BIOLOGICAL SAMPLE

(57) Abstract

The present invention provides methods and materials for detecting the presence of a fungus in a biological sample. The inventive methods and materials exploit the fact that the amino acid sequence of the saccharopine dehydrogenase molecule expressed by Candida Albicans is highly conserved in fungi. Inventive hybridization probes, nucleic acids, PCR primers, antibodies, epitopes, reagents and methods are provided.

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METHODS AND REAGENTS FOR DETECTING FUNGAL PATHOGENS IN A BIOLOGICAL SAMPLE

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to novel methods for identifying fungal pathogens in a biological sample. In particular, this invention relates to methods for screening biological samples for the presence of fungal pathogens using hybridization methods and probes capable of sensitively and specifically detecting and distinguishing nucleic acid sequences unique to fungi. Also provided are antibodies capable of binding selectively to fungal proteins.

2. Background of the Invention

Candida albicans, once considered a relatively minor fungal pathogen, has recently become a particularly serious health concern as the causative agent of candidosis (also called candidiasis). The incidence of C. albicans infections is rising rapidly with the increase in immune deficiency diseases and immunosuppressive therapy (Bodey and Fainstein, In Systemic Candidiasis, pp. 135 (Eds., Raven Press, New York 1985). Candidosis is a common nosocomial infection afflicting both immunosuppressed and postoperative patients. (Holmes, A.R., et al. Yeast-specific DNA probes and their application for the detection of Candida albicans, J. Med. Microbiol., 37:346-351 (1992)). Although candidosis is a particular concern among immunocompromised individuals, Candida infections are not limited to this group. C. albicans is the major opportunistic fungal pathogen in humans (Odds, F.C., In

Candida and candidosis, (Ed.) Leicester University Press, Leicester, United Kingdom (1989)) and is capable of establishing infection whenever the host immune system or normal flora are perturbed.

Although the C. albicans species is a particular health concern, other species of the Candida genus ar also pathogenic. The genus Candida is comprised of approximately 200 diverse yeast species classified togeth r due to their lack of a sexual cycle (Meyer et al., In Genus 4, Candida, pp. 1-12, (Ed.) N.J.W. Kreger-van Riij, Elsevier, Amsterdam (1984)). A minority of Candida species are pathogenic and 80% of the clinical isolates are eith r C. albicans or C. tropicalis (Hopfer, R.L. In Mycology of Candida Infections, G.P. Bodey, an V. Fainstein (eds.), Raven Press, New York (1985)).

In immunocompromised hosts, candidosis is a lif threatening condition. The prognosis for a patient infected with C. albicans can be improved markedly, however, with prompt antifungal treatment. Treatment may be delayed until a positive diagnosis of Candidosis is obtained since antifungal drugs are toxic. See Holmes, t al., 1992.

Diagnostic tests for the identification of C. albicans or other fungal pathogens in vivo often require complet cultural identification protocols (Musial et al., Fungal Infections of the Immunocompromised Host: Clinical and Laboratory Aspects, Clin. Microbiol. Rev. 1:349-364 (1988)). Methods currently used for the diagnosis of fungal pathogens include: cultural identification, biopsy, serodiagnosis, identification of metabolites, isoenzyme determination, pulsed field gel electrophoresis and analysis of restriction fragment length polymorphisms. Most of these methods are time consuming, laborious and

provide inconclusive results. Serodiagnosis is particularly unacceptable for the identification of candidosis, as most individuals have been exposed to Candida and therefore have circulating antibodies against Candida even in the absence of infection. Thus, serodiagnosis can only be accomplished by determining a rise in the titer for anti-Candida antibodies as compared to the titer present in the non-disease state. Such titers are generally unavailable, rendering the technique of serodiagnosis less attractive for the diagnosis of Candida infection.

Potential methods for diagnosing fungal infections through DNA screening have focused on detecting specific nucleotide sequences such as ribosomal DNA (Hopfer, R.L. et al., Detection and differentiation of fungi in clinical specimens using polymerase chain reaction (PCR) amplification and restriction enzyme analysis, J. Med. Vet. Pharm. 31:65-75 (1993)) and the P_{450} genes (Buchman, T.G. t al., Detection of surgical pathogens by in vitro DNA amplification. Part I, Rapid identification of Candida albicans by in vitro amplification of a fungal specific gene. Surgery, 108:338-347 (1990)). However, no commercial diagnostic techniques embodying methods related to the identification of these genes in biological samples are known.

One impediment to developing nucleic acid based screening techniques for Candidosis is that basic information about uniquely fungal metabolic pathways and cognate genes of C. albicans is lacking (Kurtz et al., Molecular Genetics of Candida Albicans, pp. 21-73, Kirsch, Kelly and Kurtz (eds.) CRC Press Inc. Boca Raton, Florida (1990)). The sequences of approximately forty C. albicans genes are available in computerized databases, and v ry few are involved in amino acid biosynthesis. The relativ ly

small database of genetic information available for C. albicans places limitations upon the number of DNA sequences that can be used as targets for screening probes and concomitantly reduces the likelihood of identifying a sequence unique to fungi and amenable to identification through DNA screening techniques. For example, very f w of these genes are involved in amino acid biosynthesis.

Similar impediments exist to developing immunological methods of identifying a fungus present in a biological sample. Relatively few antigenic determinants unique to fungi are known, and none are believed to have been successfully utilized as targets for antibody binding in commercially available form. Among the proteins that have been studied in C. albicans and other pathogenic fungi are the enzymes that make up the α-aminoadipate pathway for the biosynthesis of lysine. This unique pathway has been identified in Phycomycetes, Euglenids, yeasts and oth r higher fungi (Bhattacharjee, The α-aminoadipate Pathway for the Biosynthesis of Lysine in Lower Eukaryotes, CRC Critical Rev. in Microbiol. 12:131-151 (1985); Lejohn, Enzyme Regulation, Lysine Pathways and Cell Wall Structures as Indicators of Evolution in Fungi, Nature 231:164-168 (1971); and Vogel, Two Modes of Lysine Synthesis Among Lower Fungi: Evolutionary Significance, Biochim. Biophys. Acta 41:172-174 (1960)) and is present in C. albicans and other pathogenic fungi (Garrad, R. Masters Thesis, Miami University (1989) and, Garrad and Bhattacharjee, Lysine biosynthesis in selected pathogenic fungi: Characterization of lysine auxotrophs and the cloned LYS1 gene of Candida albicans, J. Bacteriol. 174:7379-7384 (1992)). Lysine is an essential amino acid for humans and animals and is synthesized by th diaminopim lic acid pathway in bacteria and plants. The α-aminoadipate pathway

consists of eight enzyme catalyzed steps; there appear to be seven free intermediates in S. cerevisiae (Bhattacharjee, The α-aminoadipate pathway for the biosynthesis of lysine in lower eukaryotes, CRC Critical Review in Microbiol. 12:131-151 (1985)). The final reversible step of the α-aminoadipate pathway is catalyzed by saccharopine dehydrogenase (EC 1.5.1.7), which is encoded by the LYS1 gene of S. cerevisiae and C. albicans, and the LYS5 gene of Y. lipolytica (Fujioka, Chemical mechanism of saccharopine dehydrogenase (NAD, L-lysine forming) as deduced from initial rate pH studies, Arch. Biochem. Biophys. 230:553-559 (1984); Garrad and Bhattacharjee, Lysine biosynthesis in selected pathogenic fungi: Characterization of lysine auxotrophs and the cloned LYS1 gene of Candida albicans, J. Bacteriol. 174:7379-7384 (1992); and Xuan et al., Overlapping reading frames at the LYS5 locus in the yeast Yarrowia lipolytica, Mol. Cell. Biol. 10:4795-4806 (1990)).

SUMMARY OF THE INVENTION

The present invention provides nucleic acid probes having nucleotide sequences that code for polypeptides that are (a) derived from saccharopine dehydrogenase expressed by wild type Candida albicans and (b) conserved among fungi, wherein the nucleic acid probes are not homologous to and do not cross react with nucleotide sequences found in the human genome. The invention also encompass s homologues of such nucleic acid probes. Additionally, the invention relates to methods for using such probes to screen biological samples for the presence of fungal pathogens. Furthermore, the invention provides a rapid method for identifying a fungus in a biological sampl based on the use of monoclonal antibodies raised to unique fungal epitopes of saccharopine dehydrogenase expressed by wild type Candida albicans.

The demand for methods for the rapid, sensitive and selective detection of fungal pathogens in biological samples and particularly for such detection of Candida albicans in biological samples increases each year. The increasing use of immunosuppressive drugs in connection with organ transplants, autoimmune diseases and cancer, taken together with the increasing number of patients suffering from acquired immunodeficiency syndrome, hav resulted in a dramatic increase in the incidenc f candidosis and other fungal infections. Because fungal infections are life threatening, physicians may prescribe antifungal drugs even in the absence of a definitive diagnosis. Due to the sometimes toxic effects of such drugs, however, their administration without such a definitive diagnosis is undesirable.

In a first aspect, this invention provides nucleic acid hybridization probes, each having a nucle tide

sequence selected from the group consisting of nucleic acid sequences that code for polypeptides that are (a) derived from saccharopine dehydrogenase expressed by wild type Candida albicans and (b) conserved among fungi, wherein the nucleic acid hybridization probes are not homologous to and do not cross react with nucleotide sequences found in the human genome. Homologues of such probes are also contemplated by the present invention. Examples of polypeptides derived from saccharopine dehydrogenase and conserved among fungi include the following:

LHLRAETKPLE (SEQ ID: 1)
LLDAGFE (SEQ ID: 2)
GLKELPE (SEQ ID: 3)
HEHIQFA (SEQ ID: 4)
LYDLEFLE (SEQ ID: 5)
GRRVAAFGF (SEQ ID: 6)
AGFAGAAIGV (SEQ ID: 7)
LVIGALGRCGSGAIDL (SEQ ID: 8)
KGGPFQEI (SEQ ID: 9)
DIFINCI (SEQ ID: 10)
IVDVSADTTNPHNP (SEQ ID: 11)
GPKLSVCSIDHLPSLLPREASE (SEQ ID: 12)
LFDKHVAR (SEQ ID: 13)

Homologues and portions of such probes are also contemplated by the present invention. For purposes of th present invention, a "portion of a probe" shall be taken to mean aprobe coding for an amino acid sequence that is a truncated version of one of the sequences provided set forth above.

In a preferred aspect, this invention provides nucleic acid hybridization probes selected from the group consisting of nucleotide sequences that code for th following polypeptides derived from saccharopine dehydrogenase:

LVIGALGRCGSGAIDL (SEQ ID: 1)
GPKLSVSIDHLPSLLPREASE (SEQ ID: 2)
DIFINCI (SEQ ID: 10) or
HEHIQFA (SEQ ID: 4)

and that are not homologous to and do not cross react with a nucleotide sequence of the human genome. Homologues of such sequences are also contemplated by th present inv ntion. Because these polypeptide sequences are

conserved among at least Candida albicans, Yarrowia lipolytica, Saccharomyces cerevisiae and Cryptococcus neoformans (conservation of SEQ ID: 2 has not been confirmed in C. neoformans) and are not known to exist in the human genome, nucleotide sequences encoding such polypeptides bind selectively and specifically to fungal nucleic acids. In a preferred embodiment, the nucleic acid hybridization probes have a sequence selected from the group consisting of:

CTTCATTTAAGAGCAGAAACTAAACCATTAGAA (SEQ ID: 14)
TTACTCGATGCTGGATTTGAA (SEQ ID: 15)
GGTTTAAAAGAATTACCTGAA (SEQ ID: 16)
CATGAACATATTCAATTTGCT (SEQ ID: 17)
TTATATGATTTAGAATTTTTAGAA (SEQ ID: 18)
GGTAGGAGAGTTGCTGCCTTTGGATTT (SEQ ID: 19)
GCTGGATTTGCTGGGGCTGCC (SEQ ID: 20)
CTTGTTATTGGTGCCTTGGGTAGATGTGGATCTGGTGCCATTGATTTA (SEQ ID: 21)
AAAGGTGGTCCATTCCAAGAAATT (SEQ ID: 22)
GATATTTTCATTAATTGTATT (SEQ ID: 23)
ATTGTTGATGTTTCTGCTGATACTACTAATCCTCATAATCCA (SEQ ID: 24)
GGTCCTAAATTATCAGTATGTTCAATTGATCATTTACCTTCTTTATTACCTAGAGAAGCTTCAGAA (SEQ ID: 25)
TTATTTGATAAACACGTTGCCAGA (SEQ ID: 26)

ATGCA GTT GAT GAA (G or A)AT (G or T)TC (SEQ ID: 27)
CAC GAG CAC ATC CAG TT(C or T) GC (SEQ ID: 28)

and the complements thereof. Fragments of the above referenced sequences are also part of the present invention as such fragments are expected to bind selectively to fungal-derived genetic material. Such sequences ar homologous to the nucleic acid sequences derived from Candida albicans that code for the conserved polypeptide sequences set forth above and are not known to cross react with sequences found in the human genome.

The invention also encompasses hybridization probes that have nucleotide sequences different from those set forth above (SEQ IDs: 14 - 28) if such probes cod for

amino acid sequences (a) derived from saccharopine dehydrogenase and (b) conserved among fungi that are not homologous to and do not cross hybridize with sequences found in the human genome.

Particularly useful embodiments of the probes may be labeled with radioactive isotopes, antigens or fluoresc nt compounds. Reagents comprising the inventive probes ar also provided. Additionally, methods of screening a biological sample for the presence of a fungal pathog n using the above referenced probes are also provided. In such methods, hybridization may optionally be conducted on filter paper or in solution. The nucleic acid to which the probe hybridizes may be isolated from a biological sample or may remain embedded in such sample. Hybridization may be detected by techniques well known in the art, such as autoradiography. In a preferred embodiment, the prob is selected from the group of preferred hybridization probes set forth above.

In another aspect, the invention provides pairs of oligonucleotides of from about 15 to about 66 nucleotides that comprise primer pairs wherein each member of the primer pair is a nucleotide sequence selected from the group consisting of nucleic acid sequences that code for polypeptides that are (a) derived from saccharopine dehydrogenase expressed by wild type Candida albicans and (b) are conserved among fungi, wherein the nucleotide sequences are not homologous to and do not cross react with nucleotide sequences found in the human genome and homologues thereof. In a preferred embodiment, each member of the primer pair is selected from the group consisting of nucleotide sequences coding for the following polypeptides:

LVIGALGRCGSGAIDL (SEQ ID: 8)
GPKLSVSIDHLPSLLPREASE (SEQ ID: 12)
DIFINCI (SEQ ID: 10) or
HEHIQFA (SEQ ID: 4)

and homologues thereof that are not homologous to and do not cross hybridize with nucleotide sequences contained in the human genome. In a particularly preferred embodiment, each member of a primer pair is selected from the gr up c o n s i s t i n g o f :

CTTGTTATTGGTGCCTTGGGTAGATGTGGATCTGGTGCCATTGATTTA (SEQ ID: 21)
GGTCCTAAATTATCAGTATGTTCAATTGATCATTTACCTTCTTTATTACCTAGAGAA GCTTCAGAA (SEQ ID: 25)

ATGCA GTT GAT GAA (G or A)AT (G or T)TC (SEQ ID: 27)

CAC GAG CAC ATC CAG TT(C or T) GC (SEQ ID: 28)

and complements thereof. Reagents comprising primer pairs selected from the group set forth above are also provided. Methods of screening biological samples for the presence of a fungal pathogen by amplifying a nucleotide sequence using the inventive primers are also provided.

In a further aspect, the invention provides methods of using antibodies to detect a fungal pathogen in a biological sample. Such methods include detecting the binding to a biological sample of antibodies that selectively bind to epitopes of saccharopine dehydrogenase expressed by wild type C. albicans but that do not bind to epitopes found in human proteins. The invention additionally provides novel antibodies for use in such assays. The antibody may be labeled and the method may comprise an enzyme linked immunosorbent assay (ELISA).

In an additional embodim nt, the invention provides novel fungal epitopes display d on saccharopin

dehydrogenase expressed by wild type Candida albicans but not displayed on mammalian proteins. These epitopes may be used to generate antibodies of the present invention. In an alternative embodiment, these epitopes may be lab l d and used to detect the presence of a fungus in a biological sample, for example, by competitively inhibiting antibody binding in a radioimmunoassay. Reagents and kits comprising the inventive antibodies and epitopes are also provided.

It is an object of the invention to provide a more rapid method for testing a biological sample for the presence of a fungal pathogen than is currently available. It is a further object of the invention to provide a sensitive method of screening biological samples for the presence of a fungal pathogen, and it is a particularly important object of the invention to provide a screening method of sufficient sensitivity to identify the presence of a fungal pathogen in a biological sample taken during the early stages of infection.

Another object of the present invention is to provide a method for identifying a fungal pathogen in a biological sample that is sufficiently specific to allow clinicians to rely upon the results in deciding whether to administer antifungal therapeutic agents and in selecting the appropriate therapeutic agent. It is a further object of the present invention to provide a relatively inexpensive method for identifying fungal pathogens in a biological sample.

It is a particular object of the present invention to provide a rapid, sensitive, selective and economical method for identifying Candida albicans in a biological sample. Employment of such a method will allow treatment of fungal inf ctions to b gin arlier than possible with current

diagnostic procedures and will therefore increase th likelihood of patient survival and shorten the duration of the fungal infection.

It is an additional object of the invention to provide nucleic acid constructs for use in screening biological samples for the presence of fungal pathogens. Another object of the invention is to provide such nucleic acid constructs that are sensitive and specific for fungal pathogens. It is a further object of the present invention to provide nucleic acid constructs for use in screening biological samples for the presence of Candida albicans.

An additional object of the invention is to provide antibodies for use in screening biological samples for the presence of fungal pathogens. Another object of the invention is to provide such antibodies that are sensitive and specific for fungal pathogens. It is a further object of the present invention to provide antibodies for use in screening biological samples for the presence of Candida albicans.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts schematically the enzymatic pathway known as the α-aminoadipate pathway.

Figure 2 provides the nucleotide sequence for the LYS 1 gene of C. albicans.

Figure 3 sets forth a comparison of the putative amino acid sequences for saccharopine dehydrogenase (or portions of that molecule) expressed by C. albicans, Y. lipolytica, S. cerevisiae and C. neoformans. Consensus information is provided only when a consensus exists between the sequences provided for all four organisms. Consensus among fewer than the four organisms exists in some cases, but is not indicated.

Figure 4 depicts the vectors and plasmids used to obtain the sequence set forth in Figure 2.

Figure 5 is a table showing transformation of various Saccharomyces strains with the LYS 1 gene from C. albicans. Those strains deficient in saccharopine dehydrogenase were transformed to prototrophy with a plasmid carrying the LYS 1 gene.

Figure 6 sets forth a comparison of the nucleotide sequ nc of a portion of the LYS 1 gene of C. albicans (nucleotides 372 - 1499) and that of a portion of the Y. lipolytica gene for saccharopine dehydrogenase (nucleotides 663 - 1757).

Figure 7 sets forth a comparison of nucleotide sequenc of a portion of the LYS 1 gene of C. albicans (nucleotid s 986 - 1324) with that of a portion of the LYS 5 gene from S. cerevisiae (nucleotides 564 - 919).

Figure 8 sets forth a comparison of the putative amino acid sequence for saccharopine dehydrogenase expressed by C. albicans with that expressed by Y. lipolytica.

Figure 9 provides the sequence of the nucleic acid fragment amplified from Cryptococcus neoformans genomic DNA using the probes and methods described in below in Example 6.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides methods and reagents for detecting fungal pathogens in biological samples. In the inventive method, the presence of a fungal pathogen may be detected using nucleic acid hybridization probes, each probe having a nucleotide sequence selected from the group consisting of nucleotide sequences that code for a polypeptide that is (a) derived from the saccharopin dehydrogenase molecule expressed by wild type Candida albicans, and (b) conserved among fungi, wherein such probes are not homologous to and do not cross react with nucleic acid sequences found in the human genome. Because humans do not express saccharopine dehydrogenase and the human genome is not known to contain a gene for this molecule, this molecule provides a unique starting point for generating hybridization probes that can be used to selectively detect fungal pathogens in a biological sample.

Homologues of such hybridization probes are also contemplated by the present invention. The presence of such fungal pathogens may also be detected using antibodies to such fungal specific C. albicans polypeptides. Th inventive methods and reagents allow for the rapid and accurate identification of the infecting organism and therefore facilitate early therapeutic intervention.

Although approximately forty genes of the C. albicans genome have been sequenced, very few of the genes involv d in amino acid biosynthesis had been sequenced prior to the current invention. The C. albicans LYS 1 gene codes for saccharopine dehydrogenase, one of the enzymes of the pathway used in fungi to generate lysine. This pathway is called the alpha aminoadipate pathway ("the a-AA pathway"); the enzymes and intermediates of this pathway are repres nted in Figure 1. The LYS1 gene was originally

cloned by Goshorn et al. (Goshorn et al. Gene isolation by complementation in Candida albicans and applications to physical and genetic mapping, Infect. and Imm. 60:876-884 (1992)). The sequence of the LYS 1 gene, determined by the present inventors, is set forth in Figure 2 (SEQ ID NO: 29). The putative amino acid sequence for the LYS1 gene, also identified by the present inventors, is also set forth in Figure 2 (SEQ ID: 30).

The present invention may be used to identify whether a subject is infected with a fungal pathogen as distinguished from a viral, bacterial or other biological pathogen. Because saccharopine dehydrogenase is not known to be expressed by bacteria or any other non-fungal organisms, it provides a unique starting point for the methods claimed herein. The invention may also be used to select appropriate antifungal drugs for use in therapeutic intervention relatively early in the disease state. It is believed that the invention is appropriate for detecting in biological samples fungal pathogens including but not limited to the following: Candida albicans, Yarrowia lipolytica and Cryptococcus neoformans. It is possible that the invention may also be appropriate for detecting Aspergillus fumigatus and Histoplasma capsulatum in a sample.

Biological samples screenable via the present invention include samples obtained from healthy subjects or those with frank or occult disease. Samples appropriate for use in the current invention should be obtained from a site on or in the body where fungi do not constitute the normal flora. The at-risk patients from which the samples are obtained include, but are not limited to mammals suffering from acquir d immune deficiency syndrome, th se under treatment with immunosuppressive drugs, postoperativ

patients and other immunocompromised patients. The samples may comprise tissues, including but not limited to swabbings from mucocutaneous membranes such as swabs from the oral cavity or the vagina, or fluids including but not limited to urine, blood, semen, cerebrospinal fluid or other bodily fluids. In a preferred embodiment, the sample is a throat swab.

The nucleic acids derived from the biological samples of the present invention may be DNA, including but not limited to cDNA, and RNA, including but not limited to mRNA. RNA derived from such samples may be particularly enriched for fungal RNAs as the fungal cells divide rapidly during infection. Thus, RNA derived from a biological sample is an important starting material for the methods of the present invention. RNA may be isolated from mixtur s of DNA and RNA by using selective exonucleases, such as DNase, and other means well known in the art. Alternatively, RNA obtained from the sample can be converted to cDNA prior to employing the inventive methods.

In the present invention, nucleic acids may be isolated from the biological samples or may remain embedded in such samples. As used herein, "nucleic acids derived from a biological sample" encompasses DNAs and RNAs either isolated from or contained in a biological sample. As us d herein, the phrases "polypeptide fragments derived from saccharopine dehydrogenase expressed by wild type *Candida albicans*" or "amino acid sequences derived from saccharopine dehydrogenase expressed by wild type *Candida albicans*" shall be taken to mean polypeptides having an amino acid sequence identical to any fragment of the saccharopine dehydrogenase protein derived from wild type *C. albicans*.

In methods where nucleic acids are first isolated from the biological sample prior to screening, the nucleic acid should be obtained in a manner so as to maintain it in an essentially undegraded state. It will be understood by those with skill in the art that by "essentially undegraded" is meant that the nucleic acid samples will be of sufficient integrity that the genes or messenger RNAs coding for saccharopine dehydrogenase in the sample will be detectable by the methods of this invention. Essentially undegraded nucleic acid is isolated by means well known to those with skill in the art. _See_, Sambrook _et al_., 1990, _Molecular Cloning: A Laboratory Manual_ (Cold Spring Harbor Press: New York), hereby incorporated by reference. Nucleic acid samples used according to the invention may be transferred directly onto a membrane, such as a nitrocellulose or a nylon membrane, or another solid support. Conversely, isolated nucleic acids may be put into solution. Britten and David [cite] describes such methods generally and is hereby incorporated by reference.

In one particularly important aspect of the invention, the nucleic acids are not isolated from the biological sample. In such methods, hybridization probes are applied directly to a biological sample in a manner known as _in situ_ hybridization. Biological samples appropriate for use in _in situ_ hybridization include tissues that may optionally be sliced or embedded in a support such as wax. The tissues may also be applied to a slide. Alternatively, in situ hybridization may be conducted _in vivo_ and hybridization determined though detection methods such as computer aided tomography. Such methods are particularly desirable as they allow for rapid processing of samples to be tested and are particularly suited to laborat ry conditions or kits for clinical use.

The present inventive methods include a method for detecting a fungal pathogen in a biological sampl by screening nucleic acids derived from the sample. As described above, appropriate samples include tissues, fluids, biopsies and the like.

In the inventive methods, the presence of a fungal pathogen in a sample may be detected with hybridization probes directed to nucleic acid sequences (and corresponding homologues) that code for polypeptid fragments of saccharopine dehydrogenase expressed by wild type Candida albicans. The hybridization probes of the present invention are not homologous to and do not cross react with nucleotide sequences of the human genome. These probes may be labeled, such as with radioactive isotopes, antigens or fluorescent compounds, to allow detection and quantification of probe hybridization.

Techniques for nucleic acid hybridization are described in Nucleic Acid Hybridization, eds. Hames, BD and Higgens, S.J., IRL Press, Oxford (1985) which is h r by incorporated by reference. In the inventive method, nucleic acids derived from a sample (whether in single stranded or double stranded form) may be transferred to a support, such as a nitrocellulose filter or nylon membran , or may be put into solution. If transferred to a support, the nucleic acid may be applied as a single sample or as a series of samples. Samples of double stranded DNA may then be denatured using a salt solution. The DNA may be processed prior to transfer onto the support, for example, by digesting the DNA with restriction enzymes and separating the resulting fragments on a gel.

The pattern of distribution of nucleic acid on th filter is s lected based on considerations such as whether

the nucleic acid bound to the filter will be hybridized with a single probe species or multiple probe species.

In one embodiment, a series of nucleic acid samples are applied to a support. These samples are bound to the support as described above. To each support, a labeled hybridization probe contained in a reagent, preferably a hybridization buffer, is applied. Such probes should be made single stranded prior to application, such as by heating briefly. The supports so treated are then incubated for approximately 6-48 hours, and washed with a moderate to high stringency wash to remove nonspecifically-hybridized probes.

The present invention contemplates the use of mixed pools of hybridization probes. Such pools would incorporate a variety of probes, such as degenerate probes or probes directed to more than one nucleotide sequence.

Hybridization is detected in a manner appropriate t the label, such as by autoradiography or fluoroscopy. Methods for detecting and quantifying hybridization are well known to those of ordinary skill in the art. In a preferred embodiment, appropriate negative (i.e. nucleic acids derived from uninfected tissue) and positive (i.e. fungal derived nucleic acids) controls are conducted to identify false negative and false positive hybridization.

Low stringency conditions are preferably employed during the annealing process to maximize hybridization of probes to homologous nucleic acid sequences. Following annealing, the filters are preferably washed under conditions of higher stringency to eliminate probes bound non-specifically.

In one embodiment, the nucleic acid sample is screened in solution. In such a method, the isolated nucleic acid may be optionally digested such as with a restriction

enzyme. Hybridization probes are added to the solution and allowed to anneal. Stringency conditions should be selected to maximize hybridization (i.e. low string ncy) and then should be raised to disrupt hybridization of probes bound to non-homologous nucleic acids. Detection and quantification of hybridization may be achieved as described above.

The results of the foregoing hybridization procedures are then used to identify the presence of a fungal pathogen in the biological sample from which the nucleic acid was obtained. This information can then be used to select appropriate therapeutic agents for treatment.

In situ hybridization methods are also encompassed by the present invention. In such methods, biological samples may be applied directly to a solid support and then treated with a labeled hybridization probe. Unannealed probes are then removed, for example, by washing. Detection of hybridization may be achieved by autoradiography, fluoroscopy or visually, such as by detecting a color change.

A particularly advantageous embodiment of the present invention would be provided by a kit comprising one or more of the following elements: a solid support, a device for obtaining a biological sample from a mucocutaneous membrane (i.e. a swab), a solution containing nucleic acid hybridization probes labeled with a visually detectable label and a washing solution. Such kits may be employed, for example, by first applying the biological sample to the support, treating the sample with a solution containing the visually detectable probe, washing away the unannealed probes and visually detecting hybridization probes bound to th biological sampl .

In an additional embodiment of the present invention, hybridization probes are used to detect restriction fragment length polymorphisms in nucleic acids isolated from a biological sample. In such method, nucleic acids are isolated from the sample and digested with a restriction endonuclease. The digested nucleic acids ar electrophoresed and blotted, as previously described. A sample known not to contain fungal nucleic acids is used as a negative control. Labeled probes having a nucleotid sequence that codes for a polypeptide that is (a) derived from saccharopine dehydrogenase expressed by Candida albicans, and (b) conserved among fungi, wherein such nucleotide sequences are not homologous to and does not cross react with nucleotide sequences found in the human genome, are then used to detect the presence f characteristic fragments of fungal nucleic acids in the biological sample.

The present inventive methods employ inventive reagents for the detection of a fungus in a sample. The reagents comprise inventive hybridization probes and appropriate hybridization buffers, which are known to those of skill in the art.

Figure 3 provides a comparison of the putative amino acid sequences for the saccharopine dehydrogenase (or a fragment of this protein) expressed by wild type versions of the following organisms: Candida albicans, Yarrowia lipolytica, Saccharomyces cerevisiae and Cryptococcus neoformans (in part). These amino acid sequences provide a starting point for generating the hybridization probes and nucleic acid primers of the present invention. Areas of consensus are provided beneath the four sequences (as the amino acid sequence for the C. neoformans runs only from residue 89 to residue 263, no consensus information is

provided for the remainder of the sequence, although conserved sequences among the remaining three organisms outside of the stretch from residue 89 - 263 are clear from Figure 3). The information provided by this figure may b used to generated hybridization probes useful for detecting a fungus in a biological sample.

The degeneracy of the genetic code requires that th probes and primers that will be useful in the pr s nt invention be described in terms of the polypeptides for which they code. Evolution results in related organisms using different codons to code for identical amino acids. Thus, the probes and primers of the present invention are those described in terms of the amino acid sequences for which they code, although exemplary sequences ar identified herein. For the purposes of the present invention, when a probe or a primer is identified by its sequence, such probe or primer shall be taken to include the complementary sequence.

Certain hybridization probes expected to be useful in detecting fungi in biological samples include th nucleotide sequences of the LYS 1 gene of Candida albicans (set forth in Fig. 2) that code for amino acid sequences conserved among fungi. The amino acid sequences which are conserved between C. albicans and Y. lipolytica, and the corresponding nucleotide sequence from C. albicans coding for those conserved sequences are set forth in Table I:

CONSERVED AMINO ACID SEQUENCE	C. albicans LYS 1 NUCLEOTIDE SEQUENCE CODING FOR AMINO ACID SEQUENCE
LHLRAETKPLE (SEQ ID: 1)	CTTCATTTAAGAGCAGAAACTAAACCATTAGAA (SEQ ID: 14)
LLDAGFE (SEQ ID: 2)	TTACTCGATGCTGGATTTGAA (SEQ ID: 15)
GLKELPE (SEQ ID: 3)	GGTTTAAAAGAATTACCTGAA (SEQ ID: 16)
HEHIQFA (SEQ ID: 4)	CATGAACATATTCAATTTGCT (SEQ ID: 17)
LYDLEFLE (SEQ ID: 5)	TTATATGATTTAGAATTTTTAGAA (SEQ ID: 18)
GRRVAAFGF (SEQ ID: 6	GGTAGGAGAGTTGCTGCCTTTGGATTT (SEQ ID: 19)
AGFAGAA (SEQ ID: 7)	GCTGGATTTGCTGGGGCTGCC (SEQ ID: 20)
LVIGALGRCGSGAIDL (SEQ ID: 8)	CTTGTTATTGGTGCCTTGGGTAGATGTGGATCTGGTGCCATTGATTTA (SEQ ID: 21)
KGGPFQEI (SEQ ID: 9)	AAAGGTGGTCCATTCCAAGAAATT (SEQ ID: 22)
DIFINCI (SEQ ID: 10)	GATATTTTCATTAATTGTATT (SEQ ID: 23)
IVDVSADTTNPHNP (SEQ ID: 11)	ATTGTTGATGTTTCTGCTGATACTACTAATCCTCATAATCCA (SEQ ID: 24)
GPKLSVCSIDHLPSLLPREASE (SEQ ID: 12)	GGTCCTAAATTATCAGTATGTTCAATTGATCATTTACCTTCTTTATTACCTAGAGAAGCTTCAGAA (SEQ ID: 25)
LFDKHVAR (SEQ ID: 13)	TTATTTGATAAACACGTTGCCAGA (SEQ ID: 26)

These amino acid sequences are highly conserved, with minor exceptions, among C. albicans, S. cerevisiae and Yarrowia lipolytica. As shown in Figure 3, many of these sequenc s are believ d to be conserved in C. neoformans as w ll,

although a full amino acid sequence for the saccharopine dehydrogenase gene for C. neoformans has not yet been suggested or shown. Degenerate probes coding for the amino acid sequences set forth above are also contemplated by th present invention of probes coding for the foregoing amino acid sequences are obviously contemplated by the present invention.

Probes preferred for use in the present invention have a maximum length of about 400 base pairs and a minimum of about 15 base pairs. In a preferred embodiment, the probes are from about 15 to about 100 base pairs long. In an especially preferred embodiment, the probes are approximately 15 - 40 base pairs long. Such sequences will hybridize selectively to fungal sequences under moderately stringent conditions as provided by the methods of the invention.

The inventive probes may be made by methods well known in the art, such as chemical synthesis. They may be synthesized manually or by machine. They may also be synthesized by recombinant methods using products incorporating viral and bacterial promoters available from Promega (Madison, Wisconsin). The probes may be single stranded or double stranded and may comprise DNA, cDNA or RNA.

The present inventive reagents may contain hybridization probes having only a single sequence, or may contain a combination of probes homologous to a variety of nucleotide sequences. The probes may be labeled, such as with radioisotopes, fluorescent compounds or antigens, to allow their detection following hybridization. In on embodiment of the present invention, an inventive reag nt contains samples of a number of different hybridization probes each sample containing a label detectable by a

different method. Use of such a reagent may, for example, be used as a control wherein a positive result would require binding of more than one type of probe to th sample.

In addition to hybridization probes, the inventive reagents may contain components including but not limited to formamide, phosphate buffers, dextran sulphate, yeast tRNA, SDS and salt. The reagents may also comprise acetyl triammonium boride, which renders the T_m of th hybridization mixture to be dependent exclusively on probe length.

The present invention also provides for the detection of fungal pathogens in biological samples following amplification of a portion of a saccharopine dehydrogenase gene, such as the LYS 1 gene. In such a method, for example, biological samples are first obtained and nucleic acids isolated as described above. Portions of genes or mRNAs coding for saccharopine dehydrogenase contained in the nucleic acid sample are then amplified by PCR (polymerase chain reaction), a technique well known to those of ordinary skill in the art. The PCR technique is described in <u>PCR Technology, Principles and Applications for DNA Amplification</u> (Erlich ed. 1989) and U.S. Pat. No. 4,683,202, the teachings of which are hereby incorporated by reference.

Inventive pairs of nucleic acid primers for use in PCR are contemplated by the present invention. Each member of such primer pair has the characteristics of the above described hybridization probes, namely, each member of the primer pair has a nucleotide sequence that is selected from the group consisting of nucleotide sequences that code for polyp ptide fragments that are (a) derived from saccharopine dehydrogenase expressed by wild type <u>Candida</u>

albicans and (b) conserved among fungi, wherein neither member of such primer pairs is homologous to nor cross reacts with nucleotide sequences found in the human genom . These inventive primer pairs, generated based on the information provided herein, including but not limited to that set forth in Table I, are employed during gene amplification.

Appropriate primer pairs are then used to amplify genetic material by well known methods. For the purposes of the present invention, a portion of a gene shall b taken to mean any portion of an entire gene, including regulatory sequences. More than one set of primer pairs may be used in the inventive method to amplify multiple gene fragments. The invention thus enables in vitro amplification of portions of fungal genes, for exampl , th LYS 1 gene, that can then be used in a screening procedur capable of identifying the presence of fungal pathogens in a biological sample.

It is also possible that the PCR method known as "Touchdown" PCR would be useful in the amplifying DNA from fungi when the primers to be used are degenerate. This technique is described in R.H. Don, et al., 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research, 19:4008 (1991) which is her by incorporated by reference.

The gene portions so amplified may be transferr d to filters or into solution in the manner described above. Reagents containing one or more hybridization probes are then applied to the samples of the amplified nucleic acids and allowed to anneal under stringency conditions as described above. Unannealed probes are then removed by washing. Hybridization of the probes to the amplified DNA

samples is then detected by means appropriate to probe label, such as by autoradiography.

The results of the hybridization experiments are then analyzed to determine the presence of a fungal pathogen in the biological sample. This information is then used in planning a course of antifungal treatment.

The primers of the present invention should be long enough to allow specific binding to fungal derived nucleic acid sequences and should have a sequence that is sufficiently homologous to a portion of the LYS1 gene to allow hybridized probes to remain bound under conditions of relatively high stringency. Each member of a primer pair to be used in connection with the present invention is selected from the group consisting of nucleic acids having nucleotide sequences coding for polypeptides that are (a) derived from saccharopine dehydrogenase that is expressed by wild type C. albicans, and (b) conserved among fungi, wherein the nucleic acids neither are homologous to nor cross-react with nucleic acids derived from mammals. Preferably, each member of the primer pairs consist of nucleic acids having at least a portion of the nucleotide sequences set forth in Table I above. More preferably, the members of the primer pairs would have the following nucleotide sequences:

ATGCA GTT GAT GAA (G or A)AT (G or T)TC (SEQ ID: 27)

CAC GAG C(A or T)C ATC CAG TTC GC (SEQ ID: 28)

The present invention also provides methods for detecting antibody binding to epitopes contained in a biological sample. Such methods entail applying an antibody, preferably a monoclonal antibody, capable of binding selectively to an epitope of C. albicans-derived saccharopine dehydrogenase and detecting selective antibody binding. Such methods include immunoblotting procedures,

wherein the proteins contained in a biological sample ar separated by electrophoresis and transferred to a support. Preferred supports include but are not limited to nitrocellulose filters and activated paper.

Proteins can be transferred to the filter by simple diffusion, vacuum assisted solvent flow or electrophoretic elution. Antibodies (either labeled or unlabeled) ar put into solution in a protein containing solvent such as BSA/PBS. The solution is then applied to the solid support harboring the blotted protein and incubated at room temperature. The blot is then washed, such as with a buffer. If the antibodies are labeled, such as with a radioactive isotope or fluorescent compound, antibody binding can then be detected. If the antibodies are unlabeled, a secondary reagent capable of disclosing bound antibody, such as avidin or streptavidin is then add d. Such secondary reagents may be enzyme labeled secondary reagents, such as those commonly utilized in enzyme link d immunosorbent assays.

The inventive antibodies may also be used to det ct a fungal pathogen in a sample by means of immunoprecipitation, such as an Odin single diffusion or Ouchterlony double diffusion test. Optionally, the proteins of the sample may be separated prior to exposur to the inventive antibodies. In an alternative embodiment, the sample may first be immunoprecipitated and subsequently separated by gel electrophoresis.

Antibodies capable of binding selectively to epitopes of Candida albicans-derived saccharopine dehydrogenase are particularly desirable for use in detecting the presence of a fungus in a biological sample as such epitopes are not known to hav counterparts among human proteins.

Antibodies, including but not limited to monoclonal antibodies, capable of selectively binding to saccharopin dehydrogenase in a biological sample can be generated through the use of hybridoma technology and related technologies well known in the art. Generation of monoclonal antibodies is described in Antibodies: A Laboratory Manual, eds. Harlow and Lane, Cold Spring Harbor, 1988, which is hereby incorporated by reference. The region of binding of such antibodies may be determined by first subjecting the target protein to enzymatic or chemical degradation, separating the fragments using electrophoresis and then immunoblotting.

In a particularly advantageous embodiment of the present invention, the inventive antibodies are employed in an enzyme linked immunosorbent assay (ELISA). In such method, the inventive antibody (the primary antibody) is anchored to a support, such as a multi-well microtiter plate. A biological sample is then added to the support, after which unbound sample is removed by washing. A second antibody to which an enzyme has been linked is applied to the support. The second antibody is one that is capable of binding to a fungal protein, though not necessarily specifically. The linked enzyme is one capable of producing a change, such as a color change, in a solution containing its substrate, the rate of color change being proportional to the enzyme concentration.

After removal of the unbound secondary antibody, a solution of the enzyme substrate is added to the support and the rate of change, such as color change, of the solution is measured. Use of such a method allows for the detection and quantification of epitopes in the sample to which the primary inventive antibody selectively binds.

In an additional aspect, the inventive methods provide a sandwich binding assay. In such an assay, the biological sample is first applied to a support, such as a filter. A inventive antibody (a primary antibody) is then appli d to the support, such as by diffusion. After unbound primary antibody is removed by washing, a second labeled antibody is applied to the support. This second labeled antibody is capable of binding to the primary antibody. Appropriat labels include but are not limited to radioactive isotopes, colored compounds and fluorescent compounds.

Unbound secondary antibody is then removed by washing. Detection of a fungal pathogen in the sample is then achieved by measuring the presence of the antibody label on the support visually, or by methods such as autoradiography or fluoroscopy.

The invention also provides novel epitopes comprising polypeptides having amino acid sequences characteristic of fungi. Such epitopes may be synthesized by methods well known in the art. Such methods include both manual and automated methods of polypeptide synthesis that may be conducted in solid phase or in solution.

In a further embodiment, the invention provides a method of detecting fungal pathogens in a biological sampl by means of a radioimmunoassay (RIA). In such a method, a sample of radioactively labeled inventive epitopes of known concentration are combined with a sample of inventiv antibodies, also of known concentration. The amount of unbound epitope contained in the solution is then measured (the first measurement). To a solution containing a known concentration of radioactively labeled inventive epitope and unlabeled inventive antibody is then added a biological sample suspect d of harboring a fungus. The amount of unbound labeled epitope in th solution is then measured

(the second measurement). The first measurement is then compared to the second measurement to detect the amount of labeled epitope displaced by epitope contained in the biological sample. These results can then be used to quantify the amount of epitope contained in the biological sample. Use of a radioimmunoassay to detect fungal pathogens in a biological sample is especially desirable as it is a particularly sensitive assay.

The invention is illustrated by the following examples.

EXAMPLE 1

The LYS1 gene of *Candida albicans* codes for saccharopine hydrogenase

A shuttle vector, ypB1041 was first constructed by Goshorn et al. (1992). The vector is a high copy number plasmid in *S. cerevisiae*, *C. albicans* and *E. coli*. The plasmid contains the 2 µm circle for replication in *S. cerevisiae*, an ARS sequence from *C. albicans* and the pBR322 origin of replication for maintenance in *E. coli*. The plasmid may be selected for in *E. coli* by resistance to ampicillin and in yeast by the presence of the URA3 sequence. This vector and constructs YpB1078 and YpB1113 were obtained from Dr. S. Scherer (Univ. of Minnesota). The vector YpB1041 is shown in Figure 4.

A *C. albicans* genomic library was created by Goshorn et al. by first partially digesting *C. albicans* DNA with Sau3A1. Fragments of 5 to 10 kb were gel purified and ligated into BamH1 restricted and alkaline phosphatase treated YpB1041. A single resulting clone, YpB1078, was confirmed to be capable of transforming a lysine auxotroph of *S. cerevisiae* and *C. albicans* to heterotrophy (Goshorn et al. Gene isolation by complementation in *Candida*

albicans and applications to physical and genetic mapping, Infect. and Imm. 60:876-884 (1992)). The plasmid was designated YpB1078 and was the original LYS1 clone.

The first subclone of LYS1, YpB1113, was created by cutting YpB1078 with Sal1 and Cla1, gel purifying the large fragment, filling in the ends with T4 DNA polymerase and deoxynucleoside triphosphates and performing a ligation of the segment. The ability of this plasmid to transform C. albicans lysine auxotrophs was demonstrated as described above and is described in more detail in Goshorn et al., 1992.

A second subclone, YpBRG2 was constructed by the present inventors by cutting YpB1113 with EcoR1 and EcoRV. The sequencing and characterization of the LYS 1 gene is described in the dissertation of Richard C. Garrad entitled "The Molecular and Functional Analysis of the LYS 1 gen of Candida albicans And Characterization of Lysine Auxotrophs of Candida Spp." completed in partial fulfillment of the requirements for Dr. Garrad's degree of Doctor of Philosophy conferred by Miami University of Ohio, the disclosure of which is hereby incorporated by referenc . This topic has also been addressed in R. Garrad et al., Molecular and Functional Analysis of the LYS1 Gene of Candida albicans, Infection and Immunity, 62:11 5027 - 5031 (1994), the disclosure of which is also hereby incorporated by reference. The subsequent 1.8 kb band was removed as a gel slice and the DNA was then electroeluted using an Elutrap system (Schleicher and Schuell, Keene, NH) or by the method described later in this section. A similar procedure was performed on pBluescript SK. The DNAs wer recovered using the method described in this section. Th 1.8 kb fragment and th EcoR1/EcoRV digested pBluescript SK w r ligated for 2 hours at room t mperature. The ligation

mix consisted of 79 μL of sterile distilled water, 10 μL 10X ligase buffer (500 mM Tris-HCl, 10 mM MgCl2 and 10 mM ATP), 10 μL 50% PEG, 1 μL 100mM dTT and 5U T4 DNA ligase. The 1.8 kb fragment was thereby ligated into the multiple cloning site of pBSSK(+/-) (Stratagene Cloning Systems, La Jolla CA).

pBSSK(+/-) is a phagemid derived from pUC19 and marketed by Stratagene Cloning Systems, La Jolla CA. The vector possesses an f1 phage origin, a ColE1 origin and T3 and T7 promoters flanking a multiple cloning site (MCS) containing 23 unique restriction sites. The vector contains a lacZ promoter for blue/white color selection. Primer sequences flank the MCS to facilitate DNA sequencing using the method of Sanger et al. (1977). The vector is shown in Figure 4.

The fragment was removed from pBSSK by cutting with BamH1 and Sal1 and ligating into a similarly cut YpB1041 using the gel purification, electroelution and ligati n procedures described later. The plasmid was designated YpBRG2 and is shown in Figure 4.

EXAMPLE 2

The ability of YpB1078 (Figure 4), YpB1113 (Figure 4) and YpBRG2 to transform S.cerevisiae Stx4-4A, (Yeast Genetics Stock Center, University of California, Berkely) was demonstrated. The ability of YpBRG2 to complement saccharopine dehydrogenase mutants of S. cerevisiae is shown in Figure 5. The amount of background, indicated by the number of colonies from cells receiving no DNA and plasmid YpB1041 is constant. Although this background is higher than ideal it is consistently at this level. S. cerevisiae STX4-4A is a point mutant and has a reversi n frequency of <7 per 10^9 cells per mL of culture.

EXAMPLE 3

Preparation of Double Stranded Template for DNA Sequencing

Double stranded templates were subjected to alkaline denaturation prior to annealing with an oligonucl otid primer. The template DNA (4µg) was diluted to 18 µL with sterile distilled water. This solution was treated with 2.0 µL of freshly made 0.2 NaOH in 0.2 mM EDTA. The mixture was incubated at room temperature for 5 minutes and then neutralized with 8.0 µL of 5 M ammonium acetate (pH 7.5). The DNA was precipitated by addition of 60 µL of 95% ethanol followed by incubation at -70°C for at least 15 minutes. The DNA was pelleted by centrifugation at 10,000xg for 10 minutes. The pellet was dried under vacuum at 45°C for 1 hour. The dried pellet was then resuspended in 7 µL of sterile distilled water.

Preparation of single stranded DNA.

Alternatively, single stranded DNA was produced to optimize the number of bases able to be read during DNA sequencing. Single stranded DNA can be produced if the sequence of interest is cloned into a suitable vector, in this case pBluescriptSK +/- and the plasmid is present in an E. coli strain which carries an F factor. Bluescript SK is a phagemid derived from pUC19 which contains f1 filamentous phage origins of replication allowing recov ry of a strand of the vector when the host strain is co-infected with a helper phage. The helper phage used in this procedure was M13KO7.

The M13KO7 must be grown from fresh plaques. The stock phage was first inoculated onto a B agar plat prepared with 1 g tryptone, 0.8 g NaCl per 100 mL of sterile distilled water sterilized by autoclaving, plus 1 mL of filt r sterilized 20% glucose plus 0.6 g agar per 100

mL. A 0.5 mL volume of mid-log phase E. coli NM522 (1 mL of overnight cells in 10 mL of LB broth incubated at 37°C for 1 hour in a shaker incubator) was added to 4 mL of B top agar, mixed and poured across the phage inoculated B agar plate. The plates were incubated for 8-12 hours at 37°C. The resulting plaques were scraped from the plat s and used to inoculate 100 mL of LB broth containing 70 μg/mL of kanamycin. The broth was incubated for 10-14 hours at 37°C. The cells were pelleted and the supernatant used for phage titering. This stock will remain viable for more than a year if stored at 4°C.

The phage was titered as follows: 100 μL of phage stock was diluted in 9.9 mL of B broth (1g tryptone, 0.8 g NaCl per 100 mL of sterile distilled water, sterilized by autoclaving, plus 1 mL of filter sterilized 20% glucose). The serial dilution was repeated 5 times and from the last two dilution tubes 100 μL was taken and added to 200 μL of log phase E. coli NM522. The phage/E. coli mixture was allowed to remain at room temperature for 5 minutes. Following this brief incubation, 4 mL of B top agar at 45°C was added to the preparation and the entire mixture poured onto a B plate. These plates were incubated at 37°C overnight. The number of plaques were counted the next day and the titer of the phage calculated.

To produce single stranded DNA the cells harboring the pBluescript vector plus the fragment of interest were grown overnight at 37°C in LBA broth with continual shaking. A 50 mL LBA broth culture in a 250 mL flask was inoculated with 1 mL of this overnight culture and incubated at 37°C for 30 minutes. Helper phage M13K07 was added to the culture at a multiplicity of infection of 20 (e.g. 200 μL of phage at a titer of $1X10^{11}$ PFU/mL). Incubation was continued for 30 minutes and then 70 μL of kanamycin (50

mg/mL) was added and incubation continued for 10-14 hours. The culture was transferred to a 50 mL Oak Ridge tube and centrifuged to remove the cells at 17,000g for 15 minutes in a Sorvall RC5 centrifuge. The supernatant was promptly removed and re-centrifuged. The volume of supernatant was then determined and for each mL of fluid 0.25 mL of a 3.5 M ammonium acetate/20% PEG 6000 solution was added, th tube was inverted to mix and then incubated on ice for 30 minutes. The mixture was centrifuged at 17,000xg for 15 minutes. The supernatant was removed and the pellet resuspended in approximately 200 μL of TE buffer (10 mM Tris.HCl pH 8.0 and 1 mM Na_2EDTA) and placed in a microfuge tube. An equal volume of equilibrated phenol/chloroform was added to the DNA, the solution was vortexed for 1 minute and then spun in a microfuge at full speed for 5 minutes. The top aqueous phase was removed and transf rred to another microfuge tube. This procedure was repeated a number of times until the interface between aqueous and non-aqueous phases was clear. An equal volume of chloroform was added to the suspension and the mixture centrifuged. The supernatant was removed into another tube and the DNA was precipitated by the addition of 100 μL 7.5 M ammonium acetate and 600 μL of 95% ethanol. The tube was incubated at 70°C for at least 30 minutes. The tube was centrifuged for 15 minutes at 4°C followed by removal of supernatant and drying of the pellet in the Speedvac. The DNA was finally resuspended in 20 μL of TE buffer. Typical yields of single stranded DNA were approximately 50 μg.

EXAMPLE 4

DNA sequencing of the LYS1 gene was performed using the methods described in the product guide of the Sequenas Version 2.0 kit (United States Bioch mical, Cleveland, OH).

The Sequenase kit employs a modification of the dideoxy chain termination method originally described by Sanger et al (1977). The DNA polymerase used in the kit is bacteriophage T7 DNA polymerase genetically engineered to remove all 3'-5' exonuclease activity.

Double and single stranded DNA templates were prepared as described above. DNA oligonucleotide primers were prepared by technical staff at Miami University on the Milligen 7500 DNA synthesizer, or at the DNA core facility of the University of Cincinnati. The oligonucleotide primers used for DNA sequencing in this study are shown in Table 2. Prior to synthesis all oligonucleotides wer analyzed for self hybridization and secondary structures using the Patterns and Loops subroutine of the DNA Star software program. The concentrations of the template DNA and oligonucleotide primers were determined from the values obtained using a Gilford UV/Visible spectrophotometer set at 260 nm absorbance. A molar ratio of 1.5/1.0 (primer/template) was used in each sequencing reaction.

Table 2. Oligonucleotide primers used in the sequencing of the *C. albicans LYS1* gene.

NAME	#	SEQUENCE (5'–3')[a]
Rev2RGBS2	1	CACAGATACTAATTAAG
RBSRG2EXT2	2	CTGAAGCTTCTCTAGG
BSRG2EXT2	3	CCTAGAGAAGCTTCAG
RRGBS2	4	GAAAATATCCAGATCCAAC
RGBS2EXT	5	GTTGATCTGGATATTTTC
RevCAN1LYS1	6	GACTCCATATCCTAATG
CAN3LYS1	7	CTTGCCAACCAGCTTGATC
RevCAN3LYS1	8	GATCAAGCTGGTTGGCAAG
NRevCAN3LYS1	9	GTACCTGAAGGTTCATG
CAN5LYS1	10	GCAGCTCTAGCTTCTAATGG
RevCAN5LYS1	11	CCATTAGAAGCTAGAGCTGC
CAN7LYS1	12	GATAATTCCGTCTAAAGT
R vCAN7LYS1	13	GACGGAATTATCTCTGTCTC
RevCAN9LYS1	14	GTGTGCACGTCCAACTC
SP2	15	AACAGCTATGACCATG
SP1	16	GTAAAACGACGGCCAGT

a The primers were designed from pBluescript sequences or from sequences of yeast DNA during the dideoxy sequencing procedure.

Annealing template and primer. Prepared doubl stranded template and single stranded template were treat d in a similar fashion except 4 μg of the former template was resuspended in 7 μL of sterile distilled water. To the 7 μL of prepared template, 1 μL of oligonucleotide primer and 2 μL of 5x reaction buffer (200 mM Tris.HCl pH 7.5, 100 mM $MgCl_2$ and 250 mM NaCl) were added and the mixture incubat d at 65°C for 2 minutes. This sample was allowed to cool to room temperature over a period of 30 minutes. During this time the 5x labeling mix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP) was diluted five fold in sterile distilled water. Four microcentrifuge tubes with 2.5 μL of each of the termination mixes, ddG (80 μM dGTP, 80 μM dATP, 80 μM dTTP, 80 μM dCTP, 50 μM NaCl and 8.0 μM ddGTP), ddA (80 μM dGTP, 80 μM dATP, 80 μM dTTP, 80 μM dCTP, 50 μM NaCl and 8.0 μM ddATP), ddC (80 μM dGTP, 80 μM dATP, 80 μM dTTP, 80 μM dCTP, 50 μM NaCl and 8.0 μM ddCTP) and ddT (80 μM dGTP, 80 μM dATP, 80 μM dTTP, 80 μM dCTP, 50 μM NaCl and 8.0 μM ddTTP) were prepared.

Labeling reaction. Once the template/primer mix was annealed 1 μL of DTT (0.1M), 2 μL of diluted labeling mix, 0.5 μL of $[\alpha\text{-}^{35}S]$ dATP (12.5uCi/μL) and 2 μL of previously diluted Sequenase Version 2.0 enzyme (1/8 in Enzyme Dilution Buffer-10mM Tris.HCl pH 7.5, 5 mM DTT and 0.5 mg/mL BSA) were added. The mixture was allowed to incubate at room temperature for 5 minutes.

Termination reactions. The termination mixes w r incubated for at least 1 minute at 37°C prior to addition of 3.5 μL of the completed labeling mix. The contents of each tube were mixed and incubated at 37°C for 5 minutes. After incubation 4 μL of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) was added to each tube. These r actions could be stored at

20°C without degradation. The termination reactions were heated to 75-80°C for at least 2 minutes before loading the sequencing gel.

Denaturing Sequencing Gel Electrophoresis. All sequencing gels were 38.5 cm x 31 cm x 0.4 mm and were electrophoresed using a BRL model S2 sequencing gel apparatus. The two glass plates which constituted the gel mold were cleaned thoroughly with detergent followed by sterile distilled water and then ethanol. Once the plates were dry a layer of Sigmacote (Sigma Laboratories, St. Louis, MO) was applied to the larger of the plates to enable easy removal after electrophoresis. Finally the large plate was given a final rinse with sterile distilled water. The two plates were placed together and separated by 0.4 mm Teflon or Kevlar spacers and the resulting gel sandwich was held together by insulating tape.

The gels were prepared from a 40% stock acrylamide solution containing 190 g acrylamide and 20 g bisacrylamide (38%:2% w/v) in a final volume of 500 mL of sterile distilled water. A stock solution of 10x Tris-Borate EDTA (TBE) running buffer was prepared by dissolving 121.1g Tris-base, 55 g boric acid and 7.4 g EDTA $Na_2.2H_2O$ in 1 liter of sterile distilled water to give a final pH of 8.3. The stock TBE was diluted to 1x when used to prepare gels and as an electrophoresis running buffer.

The preparation of 6% or 8% acrylamide gels required 15 mL or 20 mL of 40% stock acrylamide solution. In addition 50g of urea (Fisher Scientific electrophoresis grade) and 10 mL of 10x TBE buffer were mixed until the urea was completely dissolved. The volume was made up to 99 mL with sterile distilled water. Finally the acrylamide was polymerized by the addition of 1 mL of freshly prepared 10% ammonium persulfate and 20 μL of TEMED. The solution

was dispensed into the gel mold and air bubbles w re removed by gentle tapping of the glass plates prior to polymerization of the solution. The flat edges of two sharkstooth combs were inserted side by side to a depth of approximately 3 mm between the two glass plates at the top of the gel mold.

Once polymerization was complete, the combs w re removed, the tape was taken off the gel and the mold placed in the sequencing apparatus. The combs were washed and dried and placed back into the mold in the same position except now with the tips of the comb touching the acrylamide surface. The top and bottom chambers of the electrophoresis system were filled with an adequate amount of TBE running buffer (500 mL in each chamber). A needle and syringe were used to flush the formed wells of d bris. A pre-electrophoresis run was performed. Several wells were loaded with 2 μL of stop solution and the gel was run for 15-20 minutes at 1800V and 45 mA. The gel was run for the desired time with the wells loaded with samples for sequence analysis.

After electrophoresis, the gel plates were dismantled and the gel (now stuck to the small plate) was car fully lowered into a solution of 10% acetic acid/12% methanol and left to soak for at least 30 minutes. Following this period the plate was removed from the acetic acid/methanol solution. Two pieces of Whatman 3MM filter paper of larg r size than the gel were placed over the gel. The larg r glass plate was then placed on top of the filter papers to create a sandwich. After about 5 minutes, the filter paper, to which the gel was adhering, was lifted from the small glass plate. The gel was dried with heat in a Ho fer gel dry r (approximately 90 minutes) under vacuum creat d by a Savant GP100 vacuum pump. Th dried gel was expos d

to Kodak XA5 film for 24 hours at room temperature. Films were developed in Kodak D-19 for 5 minutes followed by a 1 minute wash in water and 5 minutes in Kodak dryer. The autoradiographs were rinsed in tap water for approximately 30 minutes, dried and analyzed.

Generation of a nested set of deletions using Exonuclease III digestion. The relatively large size (1.8 kb) of the insert of interest in pBluescriptSK cLYS1 makes the creation of a set of nested deletions an attractive alternative to "walking" along the insert with overlapping primers. Exonuclease III will specifically digest DNA away from a 5' protruding or blunt end restriction site. Exonuclease III will perform this digestion in a tim dependent manner, however, the sequencing primer site in the vector must be protected from digestion by the generation of a 3' overhang or by an α-phosphothioate filled end. The method used in this project made use of the Erase-a-Base System (Promega) based on the procedure developed by Henikoff (1984, 1987).

The 1.8 kb insert containing the LYS1 gene was cloned into pBluescript at the multiple cloning site. This construction allowed digestion from each side of the insert, using BamHI (Exonuclease III sensitive) and SacI (Exonuclease III resistant) from one side and ClaI (Exonuclease III sensitive) and KpnI (Exonuclease III resistant) from the other side. After digestion with one of the pairs of restriction enzymes the reaction was checked for complete digestion by agarose gel electrophoresis. If digestion was judged to be complete the mixture was extracted with 1 volume of TE saturated phenol/chloroform. The suspension was vortexed for 1 minute and centrifuged at 12,000xg for 5 minutes. The upper aqueous phase was removed to a fresh tube and 1

volume of chloroform/isoamylalcohol (24/1) was added, the mixture was then vortexed for 1 minute and centrifuged as before. The upper phase was transferred to a fresh tube and 1 volume of 2M NaCl plus 2 volumes of 95% ethanol wer added. The suspension was mixed by inverting the tube several times and the tube was incubated at 70°C for at least 30 minutes. The tube was centrifuged at 12,000xg for 10 minutes and the pellet was dried under vacuum.

The DNA pellet was dissolved in 60 μL of Exonucl as III 1x buffer (10x buffer contains 660 mM Tris.HCl pH 8.0 and 6.6 mM $MgCl_2$). While the DNA was being resuspended 7.5 μL of S1 nuclease mix was added to each of 24 microfuge tubes and kept on ice. The S1 nuclease mix was made previously (enough for 25 tubes) by adding 60U f S1 nuclease to 27 μL of S1 7.4x buffer (0.3 M potassium acetate pH 4.6, 2.5 M NaCl, 10 mM $ZnSO_4$ and 50% glycerol) plus 172 μL of sterile distilled water. The DNA was pr - warmed to 37°C and 300-500U of Exonuclease III was add d with subsequent rapid mixing. At 30 second intervals 2.5 μL samples were removed from the DNA/Exonuclease III tube and placed in the S1 nuclease mix. Once all the samples had been taken the tubes were removed from ice and placed at room temperature for 30 minutes. Following this incubation period 1 μL of S1 stop buffer (0.3 M Tris base and 0.05 M EDTA) was added to the tubes and the samples were heated at 70°C for 10 minutes to inactivate the S1 nuclease. The extent of digestions was determined by removing 2 μL samples from each time point and analyzing by agarose gel electrophoresis. The samples from each time point were transferred to 37°C and 1 μL of Klenow mix, containing 30 μL of Klenow buffer (20 mM Tris.HCl ph 8.0 amd 100 mM $MgCl_2$) and 3-5U Klenow DNA polymerase, was added to each tube. Th samples were incubated for 3 minutes

and then 1 μL of dNTP mix (0.125 M each of dATP, dCTP, dGTP and dTTP) was added followed by a further 5 minutes incubation. The samples were ligated. The tubes were transferred to room temperature and 40 μL of ligase mix was added to each mixture. The ligase mix contained 790 μL sterile distilled water, 100 μL ligase 10x buffer (500 mM Tris.HCl pH 7.6, 100 mM $MgCl_2$ amd 10 mM ATP), 100 μL 50% PEG, 10 μL 100 mM DTT and 5U T4 DNA ligase. The tubes were mixed well and incubated at room temperature for 1 hour. Following the ligation this mixture was used directly for the transformation of competent E. coli DH5α. Plasmids from E. coli DH5α transformants were prepared by mini-preparations, cut with an unique restriction enzyme and analyzed using agarose gel electrophoresis to distinguish clones of useful sizes. Plasmids of the necessary size were prepared by large scale preparation for subsequent DNA sequence analysis.

Analysis of DNA sequence data. The DNA sequence and protein data were analyzed using various programs availabl with the Genetics Computer Group software developed at the University of Wisconsin. The sequence so derived is set forth in Figure 2.

EXAMPLE 5

Base pairs 372 - 1499 of the nucleotide sequence obtained in Example 3 was compared to base pairs 663 - 1757 for the saccharopine dehydrogenase gene of Yarrowia lipolytica (this gene is named LYS5, the sequence for which was published by Xuan et al. (1990)). The comparison is set forth in Figure 6. For the portions of the two genes compared, the homology is approximately 61%. A comparison of base pairs identifi d 1 - 329 (identified as bas s 986-1315 in Figure 2) of the LYS 1 of C. albicans gene to base

pairs 564 - 919 of the gene for saccharopine dehydrogenas derived from Saccharomyces cerevisiae is set forth in Figure 7. The homology between these two gene fragments is approximately 67%. The nucleotide sequences describ d above do not appear to have significant homologs in any human gene based on a Genebank search. Thus, these regions of the LYS1 and LYS5 genes are particularly useful as starting points for constructing hybridization probes for the detection of fungal pathogens, including but not limited to C. albicans, in a biological sample.

The nucleotide sequencing data was also used to determine the putative amino acid sequence of the LYS1 gene. This sequence was compared to the predicted amino acid sequence of the LYS5 gene (Xuan et al.). This comparison is set forth in Figure 8. The sequence of th LYS 1 gene is set forth above that for the LYS 5 gene. A vertical line between two amino acids indicates compl t homology. Two points between two amino acids indicates similarity between the amino acids. A single point indicates lower similarity between the residues. A blank space between the residues shows lack of homology betwe n them.

As shown in the Figure 8, two stretches of highly conserved residues can be identified in C. albicans LYS1 and Y. lipolytica LYS5. The first stretch is amino acids 210-225 of LYS1 (corresponding to amino acids 198-213 of the Y. lipolytica gene); the second conserved region is found in amino acids 323-344 of LYS1 and amino acids 311-332 of LYS5. The region between residues 209-224 of C. albicans has an identical counterpart in the S. cerevisiae LYS1 gene.

Du to the high degree of homology between the above referenced amino acid sequ nces, these sequences ar th

most preferred starting point for generating the probes and primers of the present invention. Due to the degeneracy f the genetic code, the degree of homology between fungi of amino acid sequences may be significantly higher than the homology of the nucleic acids that encode the amino acid sequences. Thus, in the present invention, the sequences of inventive probes and primers are generally defined in terms of amino acid sequences that they encode. In preferred embodiments, the probes and primers are defined in terms of specific sequences that have shown homology between fungal species.

EXAMPLE 6

Conserved sequences identified by comparing the putative amino acid sequence of saccharopine dehydrogenase expressed by C. albicans and that expressed by Yarrowia lipolytica were used to develop PCR primers for the purpose of amplifying fungal genomic DNA from Cryptococcus neoformans (see Figure 8) (a putative amino acid sequence for S. cerevisiae saccharopine dehydrogenase was not available at the time the primers were developed). The following amino acid sequences were found to be entirely conserved between the two putative sequences: HEHIQFA and DIFINCI. Two oligonucleotides coding for these two conserved amino acid sequences were then synthesized as slightly degenerate PCR primers having the sequences set forth below (both are provided in the 5' to 3' orientation):

CAC GAG CAC ATC CAG TT(C or T) GC (SEQ ID: 27) and
ATGCA GTT GAT GAA (G or A)AT (G or T)TC (SEQ ID: 28).

These primers were used to amplify a nucleic acid 549 base pairs in length from a sample of genomic DNA isolated from Cryptococcus neoformans. The genomic DNA was isolated from

C. neoformans by the methods described by Minuth and coworkers (W. Minuth et al., Current Genetics 5:227-231 (1982)).

Amplification was conducted essentially as described in "PCR Protocols; a Guide to Methods and Applications" (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (1990), Academic Press, Inc., New York) which is h reby incorporated by reference. Specific reaction conditions for this amplification were as follows: 50 picomoles of each primer and ~10 ng of C. neoformans genomic DNA w r used. 1 - 5 units of Taq polymerase and corresponding 10X buffer was obtained from Boehringer Mannheim. A 1X solution of the Taq polymerase in buffer was made for a total volume of 100 µL. The PCR reactions were incubat d in a DNA Thermal Cycler (Perkin Elmer Cetus, Emeryvill , California) with the following cycle parameters:

One cycle was completed as follows:

stage 1 melting temperature:	94 C, one minute
annealing temperature:	37 C, one minute
extension temperature:	72 C, two minutes

Thirty cycles were then completed using the following parameters:

stage 2 melting temperature:	94 C, thirty seconds
annealing temperature:	55 C, thirty seconds
extension temperature:	72 C, thirty seconds.

The fragment so amplified was inserted into a pBluescript KS+ sequencing vector and sequenced by the dideoxynucleotide chain termination method of Sanger (F. Sanger et al., J. Mol. Biol., 94, pg. 441 (1975); F. Sang r et al., Proc. Natl. Acad. Sci. U.S.A., 74 pg. 5463 (1977)). The sequence of this 549 base pair fragment (SEQ ID: 31) is set forth in Figure 9. The underlined nucleotides at the beginning and end of the sequence represent sequences derived from the sequencing vector, pBluescript KS+. An intron is also indicated to include bases 406 - 469 in Figure 9.

It is believed that this 549 base pair nucleic acid is derived from the *C. neoformans* gene for saccharopine dehydrogenase. A putative partial amino acid sequence of the *Cryptococcus neoformans* protein is set forth in Figure 3 (SEQ ID 32). This partial sequence is based on a translation of the 549 base sequence (excluding the intron indicated in Figure 9) identified by the present inventors. This putative sequence is compared in Figure 3 with the putative amino acid sequences for *C. albicans*, *S. cerevisiae*, and *Y. lipolytica*. The intron in the 549 base pair fragment falls between the coding region for the amino acids at positions 233 and 234.

EXAMPLE 7

DNA isolated from a blood sample obtained from a patient suspected of harboring a Candida infection is subjected to PCR amplification. Appropriate primer pairs are selected from the following sequences for use in amplifying genetic material contained in the blood sampl by means of the polymerase chain reaction:

CTTGTTATTGGTGCCTTGGGTAGATGTGGATCTGGTGCCATTGATTTA (SEQ ID: 21)

GGTCCTAAATTATCAGTATGTTCAATTGATCATTTACCTTCTTTATTACCTAGAGAA GCTTCAGAA (SEQ ID: 25)

ATGCA GTT GAT GAA (G or A)AT (G or T)TC (SEQ ID: 27)

CAC GAG CAC ATC CAG TT(C or T) GC (SEQ ID: 28)

The amplified nucleic acids are transferred to a nitrocellulose filter and bound there. Fluorescently labeled nucleic acid hybridization probes homologous to at least a portion of the amplified genetic fragments ar th n applied in a hybridization buffer and are allow d to incubate with the DNA-harboring filter for 24 hours.

The filter is washed and probe binding is detected through fluoroscopy. Statistically significant probe binding is indicative of the presence of fungus in the biological sample.

Alternatively, the procedure described above may b conducted using primers included in the following description:

the nucleotide sequence of each member of the primer pair is a nucleotide sequence selected from the group consisting of nucleic acid sequences that code for polyp ptides that are (a) derived from saccharopine

dehydrogenase expressed by wild type Candida albicans and (b) are conserved among fungi, wherein the nucleotide sequences are not homologous to and do not cross react with nucleotide sequences found in the human genome and homologues thereof.

EXAMPLE 8

A biological sample is obtained from a patient suspected of harboring candidosis. Proteins from the sample are isolated, denatured and electrophoresed. The gel so produced is subjected to a procedure known to thos of skill in the art as a Western Blot. The proteins from the electrophoresis gel are vacuum eluted onto a nitrocellulose filter. To the filter is applied a sample of radiolabeled monoclonal antibody capable of binding selectively to the saccharopine dehydrogenase enzyme expressed by wild type C. albicans. The antibody is allowed to bind, after which excess antibody is removed. The filter is subjected to autoradiography and th resulting autoradiographs are interpreted to determine whether fungal proteins were present in the original biological sample.

EXAMPLE 9

A throat swab is obtained from a patient suspected of harboring a Candida infection. DNA is isolated from the sample and applied as a dot blot to a nitrocellulose filter. The filter is then treated with a high concentration salt solution and heated to bind the DNA. The filter so treated is placed in a plastic bag with prehybridization buffer.

Hybridization buffer containing radiolabeled nucleic acid hybridization probes having one of the following sequences is then applied to the filter:

CTTCATTTAAGAGCAGAAACTAAACCATTAGAA (SEQ ID: 14)
TTACTCGATGCTGGATTTGAA (SEQ ID: 15)
GGTTTAAAAGAATTACCTGAA (SEQ ID: 16)
CATGAACATATTCAATTTGCT (SEQ ID: 17)
TTATATGATTTAGAATTTTTAGAA (SEQ ID: 18)
GGTAGGAGAGTTGCTGCCTTTGGATTT (SEQ ID: 19)
GCTGGATTTGCTGGGGCTGCC (SEQ ID: 20)
CTTGTTATTGGTGCCTTGGGTAGATGTGGATCTGGTGCCATTGATTTA (SEQ ID: 21)
AAAGGTGGTCCATTCCAAGAAATT (SEQ ID: 22)
GATATTTTCATTAATTGTATT (SEQ ID: 23)
ATTGTTGATGTTTCTGCTGATACTACTAATCCTCATAATCCA (SEQ ID: 24)
GGTCCTAAATTATCAGTATGTTCAATTGATCATTTACCTTCTTTATTACCTAGAGAAGCTTCAGAA (SEQ ID: 25)
TTATTTGATAAACACGTTGCCAGA (SEQ ID: 26)

ATGCA GTT GAT GAA (G or A)AT (G or T)TC (SEQ ID: 27)
CAC GAG CAC ATC CAG TT(C or T) GC (SEQ ID: 28);

nucleic acids having nucleotide sequences that code for polypeptides that are (a) derived from saccharopine dehydrogenase expressed by wild type Candida albicans and (b) conserved among fungi, wherein the nucleic acid hybridization probes are not homologous to and do not cross react with nucleotide sequences found in the human genome; and

homologs of the sequences set forth above that will remain hybridized under relatively high stingency conditions.

The filter is incubated in the bag for approximately 24 hours. Such probes bind specifically to fungi and binding is indicative of the presence of fungal nucleic acids in the blood sample. The filter is then washed to remove unannealed probe and dried. The filter so treated is then subject d to autoradiography.

Statistically significant probe binding indicates the presence of a fungal pathogen in the sample; appropriate therapeutic intervention is then planned.

EXAMPLE 10

The procedure carried out in Example 9 is conducted in an identical fashion, with the exception that the sample utilized is a vaginal swab.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternative equivalent thereto are within the spirit or scope of the invention as set forth in the appended claims.

WHAT IS CLAIMED IS:

1. A nucleic acid hybridization probe comprising an isolated and purified nucleic acid having a nucleotide sequence selected from the group consisting of nucleotid sequences coding for a polypeptide fragment wherein the isolated and purified nucleic acid is not homologous to and does not cross react with mammalian genetic material and wherein the polypeptide fragment is (a) derived from the saccharopine dehydrogenase molecule expressed by Candida albicans and (b) conserved in fungi.

2. A nucleic acid of Claim 1 wherein the hybridization probe has a nucleotide sequence that either is identical to a sequence contained in the LYS 1 gene of Candida albicans or cross hybridizes with a portion of th LYS 1 gene of Candida albicans under conditions of high stringency.

3. A hybridization probe of Claim 1 wherein the probe has a nucleotide sequence selected from the group consisting of:

CTTCATTTAAGAGCAGAAACTAAACCATTAGAA (SEQ ID: 14)
TTACTCGATGCTGGATTTGAA (SEQ ID: 15)
GGTTTAAAAGAATTACCTGAA (SEQ ID: 16)
CATGAACATATTCAATTTGCT (SEQ ID: 17)
TTATATGATTTAGAATTTTTAGAA (SEQ ID: 18)
GGTAGGAGAGTTGCTGCCTTTGGATTT (SEQ ID: 19)
GCTGGATTTGCTGGGGCTGCC (SEQ ID: 20)
CTTGTTATTGGTGCCTTGGGTAGATGTGGATCTGGTGCCATTGATTTA (SEQ ID: 21)
AAAGGTGGTCCATTCCAAGAAATT (SEQ ID: 22)
GATATTTTCATTAATTGTATT (SEQ ID: 23)
ATTGTTGATGTTTCTGCTGATACTACTAATCCTCATAATCCA (SEQ ID: 24)
GGTCCTAAATTATCAGTATGTTCAATTGATCATTTACCTTCTTTATTACCTAGAGAAGCTTCAGAA (SEQ ID: 25)
TTATTTGATAAACACGTTGCCAGA (SEQ ID: 26)

ATGCA GTT GAT GAA (G or A)AT (G or T)TC (SEQ ID: 27)
CAC GAG CAC ATC CAG TT(C or T) GC (SEQ ID: 28).

4. A hybridization probe of Claim 1 wherein the probe is a labeled probe.

5. A hybridization probe of Claim 1 wherein the probe is labeled with a radioactive label, a fluorescent label or an antigenic label.

6. A method of screening a biological sample for the presence of a fungal pathogen, the method comprising the step of hybridizing a nucleic acid isolated from th biological sample with a hybridization probe of Claim 1.

7. A method of screening a biological sample for the presence of a fungal pathogen, the method comprising the step of hybridizing a nucleic acid isolated from th biological sample with a hybridization probe of Claim 2.

8. A method of screening a biological sample for the presence of a fungal pathogen, the method comprising the step of hybridizing a nucleic acid isolated from the biological sample with a hybridization probe of Claim 3.

9. A method of screening a biological sample for the presence of a fungal pathogen comprising detecting hybridization of a nucleic acid hybridization probe wherein the hybridization probe

(a) comprises an isolated and purified nucleic acid having a nucleotide sequence selected from the group consisting of nucleotide sequences coding for polypeptide fragments that ar (i)

derived from the saccharopine dehydrogenase molecule expressed by Candida albicans and (ii) conserved in fungi; and

(b) is not homologous to and does not cross react with mammalian genetic material.

10. The method of Claim 9 wherein the method is performed by machine.

11. The method of Claim 9 wherein the hybridization is carried out on a filter.

12. The method of Claim 9 wherein the hybridization is carried out in solution.

13. The method of Claim 9 wherein hybridization is detected by autoradiography.

14. The method of Claim 9 wherein the nucleic acid is isolated from the biological sample prior to hybridization with the hybridization probe.

15. The method of Claim 9 wherein the hybridization probe is applied directly to a biological sample.

16. The method of Claim 9 wherein the hybridization probe has a nucleotide sequence that either is identical to a sequence contained in the LYS 1 gene of Candida albicans or cross hybridizes with a portion of the LYS 1 gene of Candida albicans under conditions of high stringency.

17. The method of Claim 9 wherein the nucleotide sequence of the nucleic acid hybridization probe is selected from the group consisting of:

CTTCATTTAAGAGCAGAAACTAAACCATTAGAA (SEQ ID: 14)
TTACTCGATGCTGGATTTGAA (SEQ ID: 15)
GGTTTAAAAGAATTACCTGAA (SEQ ID: 16)
CATGAACATATTCAATTTGCT (SEQ ID: 17)
TTATATGATTTAGAATTTTTAGAA (SEQ ID: 18)
GGTAGGAGAGTTGCTGCCTTTGGATTT (SEQ ID: 19)
GCTGGATTTGCTGGGGCTGCC (SEQ ID: 20)
CTTGTTATTGGTGCCTTGGGTAGATGTGGATCTGGTGCCATTGATTTA (SEQ ID: 21)
AAAGGTGGTCCATTCCAAGAAATT (SEQ ID: 22)
GATATTTTCATTAATTGTATT (SEQ ID: 23)
ATTGTTGATGTTTCTGCTGATACTACTAATCCTCATAATCCA (SEQ ID: 24)
GGTCCTAAATTATCAGTATGTTCAATTGATCATTTACCTTCTTTATTACCTAGAGAAGCTTCAGAA (SEQ ID: 25)
TTATTTGATAAACACGTTGCCAGA (SEQ ID: 26)

ATGCA GTT GAT GAA (G or A)AT (G or T)TC (SEQ ID: 27)
CAC GAG CAC ATC CAG TT(C or T) GC (SEQ ID: 28).

18. A kit for detecting a fungal pathogen in a biological sample comprising a hybridization probe of Claim 1.

19. A kit for detecting a fungal pathogen in a biological sample comprising a hybridization probe of Claim 2.

20. A kit for detecting a fungal pathogen in a biological sample comprising a hybridization probe of Claim 3.

21. A reagent for detecting the presence of a fungal pathogen in a biological sample comprising a hybridization probe of Claim 1.

22. A reagent for detecting the presence of a fungal pathogen in a biological sample comprising a hybridization probe of Claim 2.

23. A reagent for detecting the presence of a fungal pathogen in a biological sample comprising a hybridization probe of Claim 3.

24. A pair of nucleic acid primers, each member of the pair having a nucleotide sequence selected from the group consisting of nucleotide sequences coding for a polypeptide fragments wherein the primer is not homologous to and does not cross react with mammalian genetic material and wherein the polypeptide fragment is (a) derived from the saccharopine dehydrogenase molecule expressed by _Candida albicans_ and (b) conserved in fungi.

25. A pair of nucleic acid primers, each member of the pair having a nucleotide sequence selected from the group consisting of nucleotide sequences that either are identical to a sequences contained in the LYS 1 gene of _Candida albicans_ or cross hybridize with a portion of the LYS 1 gene of _Candida albicans_ under conditions of high stringency.

26. A nucleic acid primer of Claim 24 wherein the nucleotide sequence either (a) is identical to, or (b) cross-hybridizes with under conditions of high stringency, nucleotide sequence selected from the group consisting of
CTTCATTTAAGAGCAGAAACTAAACCATTAGAA (SEQ ID: 14);
TTACTCGATGCTGGATTTGAA (SEQ ID: 15);
GGTTTAAAAGAATTACCTGAA (SEQ ID: 16);
CATGAACATATTCAATTTGCT (SEQ ID: 17);

TTATATGATTTAGAATTTTTAGAA (SEQ ID: 18);
GGTAGGAGAGTTGCTGCCTTTGGATTT (SEQ ID: 19);
GCTGGATTTGCTGGGGCTGCC (SEQ ID: 20);
CTTGTTATTGGTGCCTTGGGTAGATGTGGATCTGGTGCCATTGATTTA (SEQ ID: 21);
AAAGGTGGTCCATTCCAAGAAATT (SEQ ID: 22);
GATATTTTCATTAATTGTATT (SEQ ID: 23);
ATTGTTGATGTTTCTGCTGATACTACTAATCCTCATAATCCA (SEQ ID: 24);
GGTCCTAAATTATCAGTATGTTCAATTGATCATTTACCTTCTTTATTACCTAGAGAAGCTTCAGAA (SEQ ID: 25);
TTATTTGATAAACACGTTGCCAGA (SEQ ID: 26);
ATGCA GTT GAT GAA (G or A)AT (G or T)TC (SEQ ID: 27);
CAC GAG CAC ATC CAG TT(C or T) GC (SEQ ID: 28).

27. A method of screening a biological sample for the presence of a fungal pathogen comprising detecting hybridization of a hybridization probe to an amplified sample of genetic material, wherein the genetic material is amplified using nucleic acid primer pairs each member of the pair having a nucleotide sequence selected from the group consisting of nucleotide sequences coding for a polypeptide (a) derived from the saccharopine dehydrogenas molecule expressed by Candida albicans and (b) conserved in fungi, wherein neither member of the primer pair is homologous to or cross reacts with mammalian nucleic acids.

28. The method of Claim 27 wherein each member of the nucleic acid primer pairs has a nucleotide sequence that either is identical to a sequence contained in the LYS 1 gene of Candida albicans or cross hybridizes with a portion of the LYS 1 gene of Candida albicans under conditions of high stringency.

29. The method of Claim 27 wherein each member of th nucleic acid primer pair has a nucleic acid sequ nc selected from the group consisting of
CTTCATTTAAGAGCAGAAACTAAACCATTAGAA (SEQ ID: 14);
TTACTCGATGCTGGATTTGAA (SEQ ID: 15);
GGTTTAAAAGAATTACCTGAA (SEQ ID: 16);
CATGAACATATTCAATTTGCT (SEQ ID: 17);
TTATATGATTTAGAATTTTTAGAA (SEQ ID: 18);
GGTAGGAGAGTTGCTGCCTTTGGATTT (SEQ ID: 19);
GCTGGATTTGCTGGGGCTGCC (SEQ ID: 20);
CTTGTTATTGGTGCCTTGGGTAGATGTGGATCTGGTGCCATTGATTTA (SEQ ID: 21);
AAAGGTGGTCCATTCCAAGAAATT (SEQ ID: 22);
GATATTTTCATTAATTGTATT (SEQ ID: 23);
ATTGTTGATGTTTCTGCTGATACTACTAATCCTCATAATCCA (SEQ ID: 24);
GGTCCTAAATTATCAGTATGTTCAATTGATCATTTACCTTCTTTATTACCTAGAGAAGCTTCAGAA (SEQ ID: 25);
TTATTTGATAAACACGTTGCCAGA (SEQ ID: 26);
ATGCA GTT GAT GAA (G or A)AT (G or T)TC (SEQ ID: 27);
CAC GAG CAC ATC CAG TT(C or T) GC (SEQ ID: 28).

30. The method of Claim 27 wherein the hybridization is conducted on a filter.

31. The method of Claim 27 wherein the hybridization is conducted in solution.

32. The method of Claim 27 wherein hybridization is detected by autoradiography.

33. The method of Claim 27 wherein the method is performed by a machine.

34. A reagent for detecting the presence of a fungal pathogen in a biological sample comprising a nucleic acid primer of Claim 24.

35. A reagent for detecting the presence of a fungal pathogen in a biological sample comprising a nucleic acid primer of Claim 25.

36. A reagent for detecting the presence of a fungal pathogen in a biological sample comprising a nucleic acid primer of Claim 26.

37. A kit for detecting the presence of a fungal pathogen in a biological sample comprising a nucleic acid primer of Claim 24.

38. A kit for detecting the presence of a fungal pathogen in a biological sample comprising a nucleic acid primer of Claim 25.

39. A kit for detecting the presence of a fungal pathogen in a biological sample comprising a nucleic acid primer of Claim 26.

40. An antibody capable of binding to an epitope of saccharopine dehydrogenase expressed by wild type <u>Candida albicans</u>.

41. An antibody of Claim 41 wherein the epitope is selected from the group consisting of
LHLRAETKPLE (SEQ ID: 1)
LLDAGFE (SEQ ID: 2)
GLKELPE (SEQ ID: 3)

HEHIQFA (SEQ ID: 4)
LYDLEFLE (SEQ ID: 5)
GRRVAAFGF (SEQ ID: 6)
AGFAGAAIGV (SEQ ID: 7)
LVIGALGRCGSGAIDL (SEQ ID: 8)
KGGPFQEI (SEQ ID: 9)
DIFINCI (SEQ ID: 10)
IVDVSADTTNPHNP (SEQ ID: 11)
GPKLSVCSIDHLPSLLPREASE (SEQ ID: 12)
LFDKHVAR (SEQ ID: 13)

42. An antibody of Claim 41 wherein the antibody is labeled.

43. An antibody of Claim 43 wherein the antibody label is selected from the group consisting of a radioactive isotope, a fluorescent compound or an enzyme.

44. A method of screening a biological sample for th presence of a fungal pathogen, the method comprising detecting antibody binding to a biological sample, wher in the antibody binds selectively to saccharopine dehydrogenase expressed by wild type C. albicans.

45. The method of Claim 45 wherein the method is an enzyme linked immunosorbent assay.

46. The method of Claim 45 wherein the method is carried out by machine.

47. A kit for detecting a fungal pathogen in a biological sample wherein the kit comprises an antibody of Claim 41.

48. A reagent comprising an antibody of Claim 41.

49. An epitope comprising a polypeptide having an amino acid sequence homologous to an amino acid sequence selected from the group consisting of

LHLRAETKPLE (SEQ ID: 1)
LLDAGFE (SEQ ID: 2)
GLKELPE (SEQ ID: 3)
HEHIQFA (SEQ ID: 4)
LYDLEFLE (SEQ ID: 5)
GRRVAAFGF (SEQ ID: 6)
AGFAGAAIGV (SEQ ID: 7)
LVIGALGRCGSGAIDL (SEQ ID: 8)
KGGPFQEI (SEQ ID: 9)
DIFINCI (SEQ ID: 10)
IVDVSADTTNPHNP (SEQ ID: 11)
GPKLSVCSIDHLPSLLPREASE (SEQ ID: 12)
LFDKHVAR (SEQ ID: 13).

50. A method of using the antibody of Claim 41 comprising using the antibody to detect the presence of a fungal epitope in a biological sample wherein the antibody is used to immunoprecipitate the fungal epitope.

51. The method of Claim 45 wherein the method is a radioimmunoassay.

52. The method of Claim 45 wherein the method is an sandwich binding assay.

53. A reagent comprising the epitope of Claim 50.

54. The epitope of Claim 50 wherein the epitope is labeled.

55. A method of using the antibody of Claim 41 wherein the antibody is used in a radioimmunoassay.

56. A method of using the epitope of Claim 50 wh rein the method comprises a radioimmunoassay.

57. A method of using the hybridization probe of Claim 1, wherein the probe is used to identify restriction fragment length polymorphisms in nucleic acid isolated from a biological sample and digested.

α-Ketoglutarate + Acetyl CoA --HSY--> Homocitrate --HCD-->

Homoaconitate --HAH--> Homoisocitrate --HDH-->

α-Ketoadipate --AAT--> α-Aminoadipate

--AAR--> α-aminoadipic-gamma-semialdehyde --SRD-->

saccharopine --SDH--> Lysine + α-ketoglutarate

FIGURE 1

FUNGAL α -AMINOADIPATE PATHWAY

```
           BamHI                    Ec RI
             /                       /
     CTAGTGGATCCCCCGGGCTGCAGGAATTCTTCTTCTTTTCTCCGTCTGAC
1    ---------+---------+---------+---------+---------+
     GATCACCTAGGGGGCCCGACGTCCTTAAGAAGAAGAAAAGAGGCAGACTG

     TCATTTTAATCGTCTGGTGGCTGGTGGCTGGTGGCTGGCGGCGGCACGGG
51   ---------+---------+---------+---------+---------+
     AGTAAAATTAGCAGACCACCGACCACCGACCACCGACCGCCGCCGTGCCC

     CAGCGGCAGCGGTGATGAGTGTGAGTTCCTTAATTATCGCCGCATGTTAT
101  ---------+---------+---------+---------+---------+
     GTCGCCGTCGCCACTACTCACACTCAAGGAATTAATAGCGGCGTACAATA

     TACTCACTCACTCACAAACACTTTAGACGGAATTATCTCTGTCTCTCTCT
151  ---------+---------+---------+---------+---------+
     ATGAGTGAGTGAGTGTTTGTGAAATCTGCCTTAATAGAGACAGAGAGAGA

     CTCTGTCTCTCTCTCTTTCTCACTTAGAGAATATATAAACCACATTACAA
201  ---------+---------+---------+---------+---------+
     GAGACAGAGAGAGAGAAAGAGTGAATCTCTTATATATTTGGTGTAATGTT

     TTCATTTATTCTACATTGAACAATTTGAATGAAAAAAAAAAAAACATTTT
251  ---------+---------+---------+---------+---------+
     AAGTAAATAAGATGTAACTTGTTAAACTTACTTTTTTTTTTTTTGTAAAA

     ATACCTTTACTTCTTACTTCTTTCTAATAATCAACTATACTAGCTAACTC
301  ---------+---------+---------+---------+---------+
     TATGGAAATGAAGAATGAAGAAAGATTATTAGTTGATATGATCGATTGAG

     ATATACTAATTATGTCTAAATCACCAGTTATTCTTCATTTAAGAGCAGAA
351  ---------+---------+---------+---------+---------+
     TATATGATTAATACAGATTTAGTGGTCAATAAGAAGTAAATTCTCGTCTT
                          M  S  K  S  P  V  I  L  H  L  R  A  E

     ACTAAACCATTAGAAGCTAGAGCTGCTTTAACTCCTTCTACTACTAAACA
401  ---------+---------+---------+---------+---------+
     TGATTTGGTAATCTTCGATCTCGACGAAATTGAGGAAGATGATGATTTGT
     T  K  P  L  E  A  R  A  A  L  T  P  S  T  T  K  Q
```

FIGURE 2

```
     ATTACTCGATGCTGGATT TGAAATTTATGTTGAAGAATCTTCTCAATCTA
451  ---------+---------+---------+---------+---------+
     TAATGAGCTACGACCTAAACTTTAAATACAACTTCTTAGAAGAGTTAGAT
      L  L  D  A  G  F  E  I  Y  V  E  E  S  S  Q  S  T

     CTTTTGATATTAAAGAATATGAAGCTGTTGGTGCTAAAATAGTACCTGAA
501  ---------+---------+---------+---------+---------+
     GAAAACTATAATTTCTTATACTTCGACAACCACGATTTTATCATGGACTT
       F  D  I  K  E  Y  E  A  V  G  A  K  I  V  P  E

     GGTTCATGGAAAACTGCTCCTAAAGAGAGAATTATTTTTGGTTTAAAAGA
551  ---------+---------+---------+---------+---------+
     CCAAGTACCTTTTGACGAGGATTTCTCTCTTAATAAAAACCAAATTTTCT
     G  S  W  K  T  A  P  K  E  R  I  I  F  G  L  K  E

     ATTACCTGAAAATGAAACTTTCCCATTAATTCATGAACATATTCAATTTG
601  ---------+---------+---------+---------+---------+
     TAATGGACTTTTACTTTGAAAGGGTAATTAAGTACTTGTATAAGTTAAAC
      L  P  E  N  E  T  F  P  L  I  H  E  H  I  Q  F  A

     CTCATTGTTATAAAGATCAAGCTGGTTGGCAAGATGTTTTAAAAAGATTC
651  ---------+---------+---------+---------+---------+
     GAGTAACAATATTTCTAGTTCGACCAACCGTTCTACAAAATTTTTCTAAG
       H  C  Y  K  D  Q  A  G  W  Q  D  V  L  K  R  F

     CCACAAGGTAATGGTATATTATATGATTTAGAATTTTTAGAAAATGATCA
701  ---------+---------+---------+---------+---------+
     GGTGTTCCATTACCATATAATATACTAAATCTTAAAAATCTTTTACTAGT
     P  Q  G  N  G  I  L  Y  D  L  E  F  L  E  N  D  Q

     AGGTAGGAGAGTTGCTGCCTTTGGATTTTATGCTGGATTTGCTGGGGCTG
751  ---------+---------+---------+---------+---------+
     TCCATCCTCTCAACGACGGAAACCTAAAATACGACCTAAACGACCCCGAC
      G  R  R  V  A  A  F  G  F  Y  A  G  F  A  G  A  A

     CCATTGGGGTATTAGATTGGAGTTTTAAACAATTGAATGGTAATACTAAA
801  ---------+---------+---------+---------+---------+
     GGTAACCCCATAATCTAACCTCAAAATTTGTTAACTTACCATTATGATTT
       I  G  V  L  D  W  S  F  K  Q  L  N  G  N  T  K

     GGTACTAAAGGTGAAGGTGAAGGTGGTGAATTACCTGGGGTGACTCCATA
851  ---------+---------+---------+---------+---------+
     CCATGATTTCCACTTCCACTTCCACCACTTAATGGACCCCACTGAGGTAT
     G  T  K  G  E  G  E  G  G  E  L  P  G  V  T  P  Y
```

FIGURE 2 (CONT)

```
     TCCTAATGAAAATGAATTAATTAAAGATGTTAAAATTGAATTAGAAAAAG
901  ---------+---------+---------+---------+---------+
     AGGATTACTTTTACTTAATTAATTTCTACAATTTTAACTTAATCTTTTTC
      P  N  E  N  E  L  I  K  D  V  K  I  E  L  E  K  A

     CTTTAACTAAAAATGGGGGTCAATATCCTAAATGTCTTGTTATTGGTGCC
951  ---------+---------+---------+---------+---------+
     GAAATTGATTTTTACCCCCAGTTATAGGATTTACAGAACAATAACCACGG
      L  T  K  N  G  G  Q  Y  P  K  C  L  V  I  G  A

     TTGGGTAGATGTGGATCTGGTGCCATTGATTTATTTAAAAAAATTGGTAT
1001 ---------+---------+---------+---------+---------+
     AACCCATCTACACCTAGACCACGGTAACTAAATAAATTTTTTTAACCATA
     L  G  R  C  G  S  G  A  I  D  L  F  K  K  I  G  I

     CCCTGATGATAATATTGCTAAATGGGATATGGCTGAAACTGCTAAAGGTG
1051 ---------+---------+---------+---------+---------+
     GGGACTACTATTATAACGATTTACCCTATACCGACTTTGACGATTTCCAC
      P  D  D  N  I  A  K  W  D  M  A  E  T  A  K  G  G

     GTCCATTCCAAGAAATTGTTGATCTGGATATTTTCATTAATTGTATTTAT
1101 ---------+---------+---------+---------+---------+
     CAGGTAAGGTTCTTTAACAACTAGACCTATAAAAGTAATTAACATAAATA
      P  F  Q  E  I  V  D  L  D  I  F  I  N  C  I  Y

     TTATCTAAACCAATCCCACCATTTATTAATAAAGAAATTTTGAATAATGA
1151 ---------+---------+---------+---------+---------+
     AATAGATTTGGTTAGGGTGGTAAATAATTATTTCTTTAAAACTTATTACT
     L  S  K  P  I  P  P  F  I  N  K  E  I  L  N  N  E

     AAATAGAAAATTGACTACTATTGTTGATGTTTCTGCTGATACTACTAATC
1201 ---------+---------+---------+---------+---------+
     TTTATCTTTTAACTGATGATAACAACTACAAAGACGACTATGATGATTAG
      N  R  K  L  T  T  I  V  D  V  S  A  D  T  T  N  P

     CTCATAATCCAATCCCAGTATATGAAATTGCTACAGTTTTCAATGAACCA
1251 ---------+---------+---------+---------+---------+
     GAGTATTAGGTTAGGGTCATATACTTTAACGATGTCAAAAGTTACTTGGT
      H  N  P  I  P  V  Y  E  I  A  T  V  F  N  E  P
```

FIGURE 2 (CONT.)

```
     ACCGTTGAAGTTAAACTTGATAAAGGTCCTAAATTATCAGTATGTTCAAT
1301 ---------+---------+---------+---------+---------+
     TGGCAACTTCAATTTGAACTATTTCCAGGATTTAATAGTCATACAAGTTA
     T  V  E  V  K  L  D  K  G  P  K  L  S  V  C  S  I

                                HindIII
                                 /
     TGATCATTTACCTTCTTTATTACCTAGAGAAGCTTCAGAATTTTTTGCTA
1351 ---------+---------+---------+---------+---------+
     ACTAGTAAATGGAAGAAATAATGGATCTCTTCGAAGTCTTAAAAAACGAT
      D  H  L  P  S  L  L  P  R  E  A  S  E  F  F  A  K

     AAGATTTAATGCCATCATTATTGGAATTACCAAATAGAGATACTTCTCCA
1401 ---------+---------+---------+---------+---------+
     TTCTAAATTACGGTAGTAATAACCTTAATGGTTTATCTCTATGAAGAGGT
       D  L  M  P  S  L  L  E  L  P  N  R  D  T  S  P

     GTATGGGTTAGAGCTAAACAATTATTTGATAAACACGTTGCCAGACTTGA
1451 ---------+---------+---------+---------+---------+
     CATACCCAATCTCGATTTGTTAATAAACTATTTGTGCAACGGTCTGAACT
     V  W  V  R  A  K  Q  L  F  D  K  H  V  A  R  L  D

     TAAAGAGTAGTAGTAGGTTTACAAGTCAAGTAAATGTGTTTAATAAATAT
1501 ---------+---------+---------+---------+---------+
     ATTTCTCATCATCATCCAAATGTTCAGTTCATTTACACAAATTATTTATA
      K  E  *  *  *

                       Poly A
                 *****************                 ***
     TTTATTAAATCTTTTATTTTATTTTATTTCATTTCATTTCTTAATTAGTA
1551 ---------+---------+---------+---------+---------+
     AAATAATTTAGAAAATAAAATAAAATAAAGTAAAGTAAAGAATTAATCAT

                           ****
     TCTGTGTATATTGGGATCTATTAGTAAAATAGTAGCACTATTATTATTCT
1601 ---------+---------+---------+---------+---------+
     AGACACATATAACCCTAGATAATCATTTTATCATCGTGATAATAATAAGA

                  ***
     AATGTTACACTAACTTTTCTTTTCTTTTTAATATTATTCTTTTTTGATTT
1651 ---------+---------+---------+---------+---------+
     TTACAATGTGATTGAAAAGAAAAGAAAAATTATAATAAGAAAAAACTAAA

                                     PolyA
                                     ******
     CTTACCCTTTTTATTCTTTTCACCTTGCATTATATTTTTAATTTCTTCAC
1701 ---------+---------+---------+---------+---------+
     GAATGGGAAAAATAAGAAAAGTGGAACGTAATATAAAAATTAAAGAAGTG
```

FIGURE 2 (CONT)

```
                                          EcoRV   HindIII      SalI
                                              /   /               /
     CATCAGTTTCATATTCAGATTCACTAGGGATATCAAGCTTATCGATACCG
1751 ---------+---------+---------+---------+---------+
     GTAGTCAAAGTATAAGTCTAAGTGATCCCTATAGTTCGAATAGCTATGGC

     TCGACC
1801 ------ 1806
     AGCTGG
```

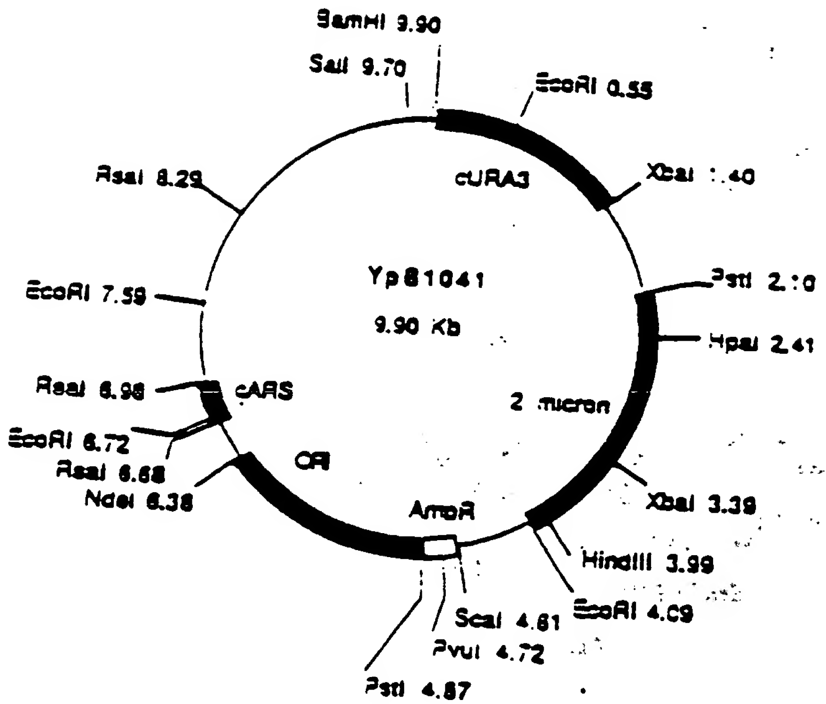
FIGURE 2 (CONT)

```
C. albicans     1    MSKSPVILHL RAETKPLEAR AALTPSTTKQ LLDAGFEIYV
S. cerevisiae        ..MAAVTLHL RAETKPLEAR AALTPTTVKK LIAKGFKIYV
Y. lipolytica        .MTAPVKLHL RAETKPLEHR SALTPTTTRK LLDAGFEVFV
C. neoformans        .......... .......... .......... ..........
Consensus            ---------- ---------- ---------- ----------

C. albicans     41   EESSQSTFDI KEYEAVGAKI VPEGSWKTAP KERIIFGLKE
S. cerevisiae        EDSPQSTFNI NEYRQAGAII VPAGSWKTAP RDRIIIGLKE
Y. lipolytica        EKSPLRIFDD QEFVDVGATL VEEGSWVSAP EDRMIIGLKE
C. neoformans        .......... .......... .......... ..........
Consensus            ---------- ---------- ---------- ----------

C. albicans     81   LPENETFPLI HEHIQFAHCY KDQAGWQDVL KRFPQGNGIL
S. cerevisiae        MPETDTFPLV HEHIQFAHCY KDQAGWQNVL MRFIKGAGTL
Y. lipolytica        LPE.ESFPLS HEHIQFAHCY KDQGGWKDVL SRFPAGNGTL
C. neoformans        .......... HEHIQFAHCY KQQAGWNDVL RRFAQGKGTL
Consensus            ---------- HEHIQFAHCY K-Q-GW--VL -RF--G-G-L

C. albicans     121  YDLEFLENDQ G.RRVAAFGF YAGFAGAAIG VLDWSFKQLN
S. cerevisiae        YDLEFLENDQ G.RRVAAFGF YAGFAGAALG VRDWAFKQ..
Y. lipolytica        YDLEFLEDDN G.RRVAAFGF HAGFAGAAIG VETWAFQQ..
C. neoformans        YDLEFLEDPV SHRRVAAFGF HAGFAGAAAG ALAFAAQQ..
Consensus            YDLEFLE--- --RRVAAFGF -AGFAGAA-G -------Q--

C. albicans     161  GNTKGTKGEG EGGELPGVTP YPNENELIKD VKIELEKALT
S. cerevisiae        .......THS DDEDLPAVSP YPNEKALVKD VTKDYKEALA
Y. lipolytica        .......THP DSENLPGVSA YPNETELVDK IKKDLAAAVE
C. neoformans        .......TQN GQGKLGELKP YPNEGEMVKE VSEALEG..T
Consensus            ---------- ----L----- YPNE------ ----------

C. albicans     201  KNGGQYPKCL VIGALGRCGS GAIDLFKKIG IPDDNIAKWD
S. cerevisiae        .TGARKPTVL IIGALGRCGS GAIDLLHKVG IPDANILKWD
Y. lipolytica        K.GSKLPTVL VIGALGRCGS GAIDLARKVG IPEENIIRWD
C. neoformans        KEGKKGVKVL IIGALGRCGS GAVDLFRKAG VAEENIVKWD
Consensus            --G------L -IGALGRCGS GA-DL--K-G ----NI--WD

C. albicans     241  MAETAKGGPF QEIVDLDIFI NCIYLSKPIP PFINKEILNN
S. cerevisiae        IKETSRGGPF DEIPQADIFI NCIYLSKPIA PFTNMEKLNN
Y. lipolytica        MNETKKGGPF QEIADADIFI NCIYLSQPIP PFINYDLLNK
C. neoformans        MAETAKGGPF PEILDVDIFI NC........ ..........
Consensus            --ET--GGPF -EI---DIFI NC-------- ----------
```

FIGURE 3
PUTATIVE AMINO ACID SEQUENCES FOR SACCHAROPINE DEHYDROGENASE EXPRESSED BY C. albicans, S. cerevisiae, Y. lipolytica and C. neoformans

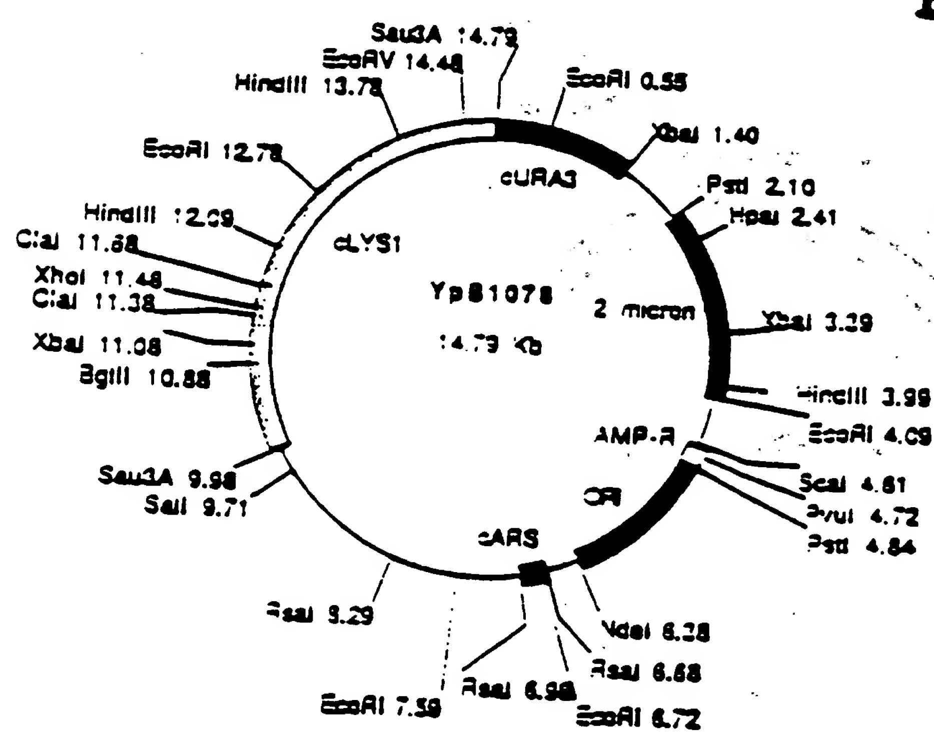
C. albicans	281	ENRKLTTIVD	VSADTTNPHN	PIPVYEIATV	FNEPTVEVKL
S. cerevisiae		PNRRLRTVVD	VSADTTNPHN	PIPIYTVATV	FNKPTVLVPT
Y. liplytica		ETRKLSVIVD	VSADTTNPHN	PVPVYTIATT	FDHPTVPVET
C. ne formans		....	....	....	....
Consensus		----------	----------	----------	----------
C. albicans	321	DKGPKLSVCS	IDHLPSLLPR	EASEFFAKDL	MPSLLELPNR
S. cerevisiae		TVGPKLSVIS	IDHLPSLLPR	EASEFFSHDL	LPSLELLPQR
Y. lipolytica		TAGPKLSVCS	IDHLPSLLPR	EASEAFSEAL	LPSLLQLPQR
C. neoformans		....	....	....	....
Consensus		----------	----------	----------	----------
C. albicans	361	DTSPVWVRAK	QLFDKHVARL	DKE...	
S. cerevisiae		KTAPVWVRAK	KLFDRH CARV	KRSSRL	
Y. lipolytica		DTAPVWTRAK	ALFDKHVLRI	GE....	
C. neoformans		....	....	......	
Consensus		----------	----------	------	

FIGURE 3 (continued)
PUTATIVE AMINO ACID SEQUENCES FOR SACCHAROPINE DEHYDROGENASE EXPRESSED BY C. albicans, S. cerevisiae, Y. lipolytica and C. neoformans

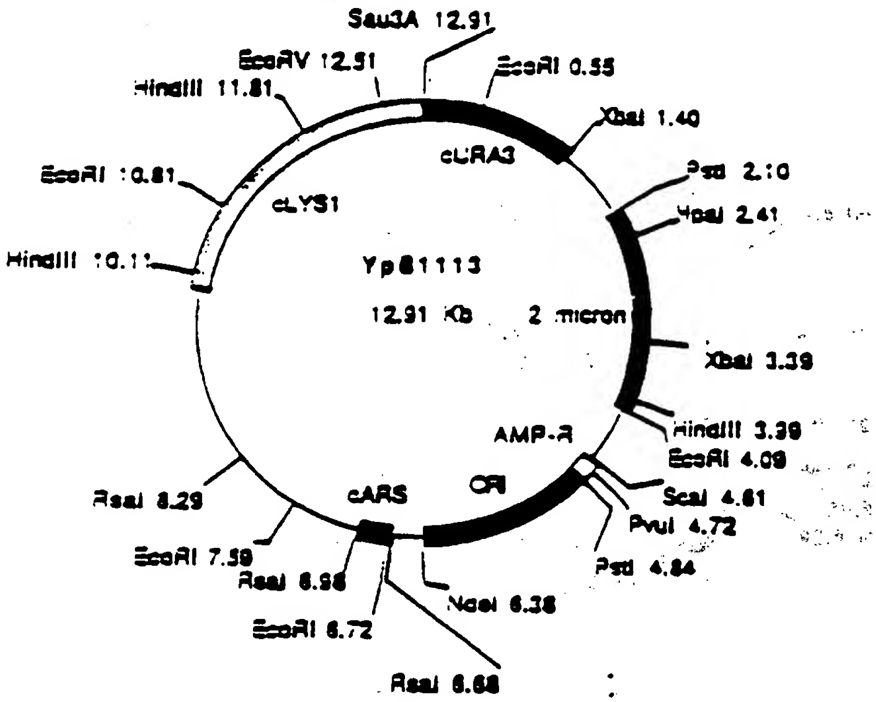

BamHI 9.90
SalI 9.70
EcoRI 0.55
XbaI 1.40
RsaI 8.29
cURA3
Yp81041
9.90 Kb
EcoRI 7.59
PstI 2.10
HpaI 2.41
RsaI 6.98
cARS
2 micron
EcoRI 6.72
RsaI 6.68
NdeI 6.38
ORI
AmpR
XbaI 3.39
HindIII 3.99
EcoRI 4.09
ScaI 4.61
PvuI 4.72
PstI 4.87

A

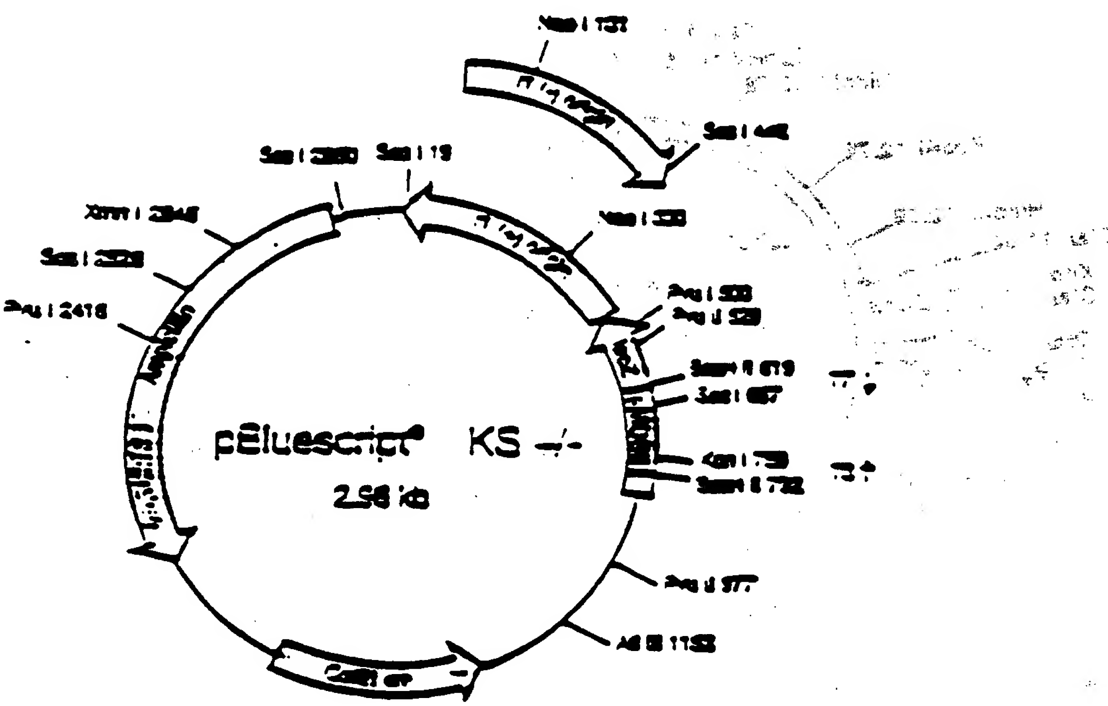
FIGURE 4


Sau3A 14.79
EcoRV 14.48
HindIII 13.78
EcoRI 0.55
XbaI 1.40
EcoRI 12.78
cURA3
PstI 2.10
HpaI 2.41
HindIII 12.09
ClaI 11.68
cLYS1
XhoI 11.48
ClaI 11.38
Yp81078
14.79 Kb
2 micron
XbaI 3.39
XbaI 11.08
BglII 10.88
HindIII 3.99
EcoRI 4.09
AMP-R
ORI
Sau3A 9.98
SalI 9.71
ScaI 4.61
PvuI 4.72
PstI 4.84
cARS
RsaI 8.29
NdeI 6.38
RsaI 6.68
RsaI 6.98
EcoRI 7.59
EcoRI 6.72

B



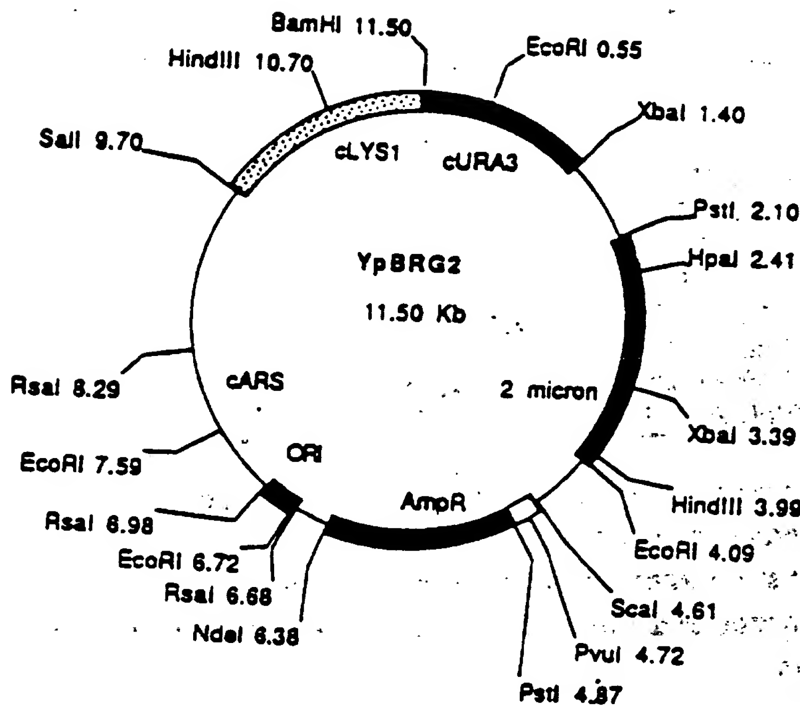
A



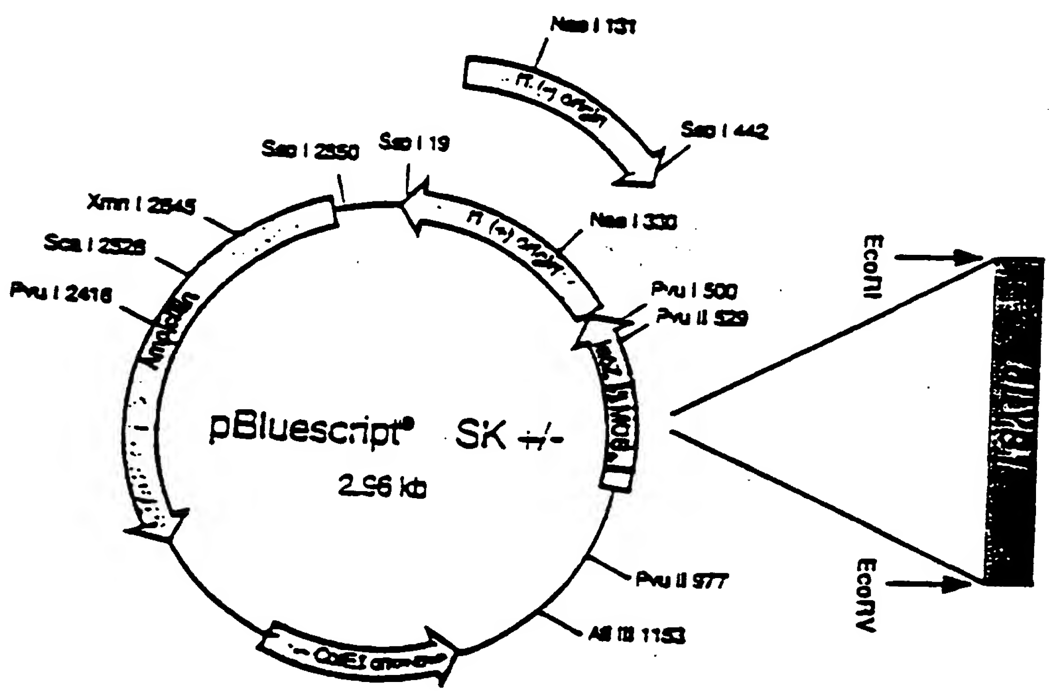
B

FIGURE 4 (CONT.)

FIGURE 4 (CONT)



A



Saccharopine dehydrogenase activity of wild type, *lys1* mutants and *Lys1*+ transformed strains of *S. cerevisiae*.

Organism and Strain	Lysine genotype	Saccharopine dehydrogenase activity[a]
S. cerevisiae		
WT	*LYS1*	0.49 (±0.01)
STX4-4A	*lys1*	0.08 (±0.01)
STX4-4A-8 (YpB 1078)[b]	*Lys1*+	0.26 (±0.05)
STX4-4A-8 (YpB 1078)[c]	*lys1*	0.06 (±0.01)
STX4-4A-3 (YpB RG2)[b]	*Lys1*+	0.89 (±0.22)
STX4-4A-3 (YpB RG2)[c]	*lys1*	0.09 (±0.01)
STX4-4A-4 (YpB RG2)[b]	*Lys1*+	0.69 (±0.22)
STX4-4A-4 (YpB RG2)[c]	*lys1*	0.14 (±0.01)
STX4-4A-5 (YpB RG2)[b]	*Lys1*+	0.74 (±0.19)
STX4-4A-5 (YpB RG2)[c]	*lys1*	0.13 (±0.01)

a Saccharopine dehydrogenase specific activity expressed as change in absorbance at 340 nm per min. per mg. protein.

b Mutant strain transformed with stated plasmid.

c Transformed strain which lost plasmid after growth on non-selective medium.

FIGURE 5

```
 372 CACCAGTTATTCTTCATTTAAGAGCAGAAACTAAACCATTAGAAGCTAGA 421
1757 CACCAGTGAAGCTCCATCTCCGAGCCGAGACCAAGCCTCTCGAGCACCGA 1708

 422 GCTGCTTTAACTCCTTCTACTACTAAACAATTACTCGATGCTGGATTTGA 471
1707 TCTGCTCTCACGCCTACTACCACCCGAAAGCTGCTTGATGCTGGATTCGA 1658

 472 AATTTATGTTGAAGAATCTTCTCAATCTACTTTTGATATTAAAGAATATG 521
1657 GGTCTTTGTGGAGAAGTCTCCCCTTCGAATCTTCGACGACCAGGAGTTTG 1608

 522 AAGCTGTTGGTGCTAAAATAGTACCTGAAGGTTCATGGAAAACTGCTCCT 571
1607 TCGATGTCGGAGCCACTCTTGTCGAGGAGGGCTCTTGGGTCTCTGCCCCC 1558

 572 AAAGAGAGAATTATTTTTGGTTTAAAAGAATTACCTGAAAATGAAACTTT 621
1557 GAGGACCGAATGATTATTGGTCTTAAGGAGCTGCCTG...AGGAATCTTT 1511

 622 CCCATTAATTCATGAACATATTCAATTTGCTCATTGTTATAAAGATCAAG 671
1510 CCCTCTGTCTCACGAGCACATCCAGTTTGCTCACTGCTACAAGGATCAGG 1461
```

FIGURE 6

```
 672 CTGGTTGGCAAGATGTTTTAAAAAGATTCCCACAAGGTAATGGTATATTA 721
1460 GCGGATGGAAGGACGTTCTGAGCCGATTCCCCGCAGGAAACGGAACTCTG 1411

 722 TATGATTTAGAATTTTTAGAAAATGATCAAGGTAGGAGAGTTGCTGCCTT 771
1410 TACGACCTTGAGTTCCTGGAGGATGACAATGGACGACGAGTTGCCGCCTT 1361

 772 TGGATTTTATGCTGGATTTGCTGGGGCTGCCATTGGGGTATTAGATTGGA 821
1360 TGGCTTCCACGCTGGATTCGCCGGTGCCGCCATCGGTGTCGAGACTTGGG 1311

 822 GTTTTAAACAATTGAATGGTAATACTAAAGGTACTAAAGGTGAAGGTGAA 871
1310 CCTTCCAGCA.....................GACCCACCCCGACAGCGAA 1282

 872 GGTGGTGAATTACCTGGGGTGACTCCATATCCTAATGAAAATGAATTAAT 921
1281 ......AACCTGCCCGGTGTCTCTGCCTATCCCAATGAGACCGAGCTTGT 1238

 922 TAAAGATGTTAAAATTGAATTAGAAAAAGCTTTAACTAAAAATGGGGGTC 971
1237 CGACAAGATTAAGAAGGATCTTGCCGCTGCT....GTTGAGAAGGGCTCC 1192

 972 AATATCCTAAATGT.CTTGTTATTGGTGCCTTGGGTAGATGTGGATCTGG 1020
1191 AAGCTCCCTACCGTCCTGGTGATTGGTGCTCTTGGCCGATGTGGATCCGG 1142

1021 TGCCATTGATTTATTTAAAAAAATTGGTATCCCTGATGATAATATTGCTA 1070
1141 TGCCATTGATCTGGCCCGAAAGGTCGGTATCCCCGAAGAGAACATCATTC 1092

1071 AATGGGATATGGCTGAAACTGCTAAAGGTGGTCCATTCCAAGAAATTGTT 1120
1091 GATGGGACATGAACGAGACCAAGAAGGGTGGACCCTTCCAAGAGATTGCT 1042

1121 GATCTGGATATTTTCATTAATTGTATTTATTTATCTAAACCAATCCCACC 1170
1041 GACGCGGATATCTTCATCAACTGCATCTACCTGTCTCAGCCCATTCCTCC 992

1171 ATTTATTAATAAAGAAATTTTGAATAATGAAAATAGAAAATTGACTACTA 1220
 991 TTTCATCAACTACGATCTGCTCAACAAGGAGACCCGAAAGCTCAGTGTCA 942

1221 TTGTTGATGTTTCTGCTGATACTACTAATCCTCATAATCCAATCCCAGTA 1270
 941 TTGTCGACGTCTCTGCTGACACCACCAACCCCCACAACCCTGTCCCCGTG 892

1271 TATGAAATTGCTACAGTTTTCAATGAACCAACCGTTGAAGTTAAACTTGA 1320
 891 TACACAATTGCTACCACGTTCGACCATCCCACCGTGCCTGTTGAGACCAC 842

1321 TAAAGGTCCTAAATTATCAGTATGTTCAATTGATCATTTACCTTCTTTAT 1370
```

FIGURE 6 (CONT.)

```
 841 TGCTGGCCCCAAGCTGTCCGTGTGCTCGATCGACCACCTGCCCTCTCTTC 792

1371 TACCTAGAGAAGCTTCAGAATTTTTTGCTAAAGATTTAATGCCATCATTA 1420
 791 TGCCGCGAGAGGCTTCCGAGGCCTTTTCTGAGGCTCTGCTGCCTTCTCTC 742

1421 TTGGAATTACCAAATAGAGATACTTCTCCAGTATGGGTTAGAGCTAAACA 1470
 741 CTGCAGCTTCCTCAGCGAGACACTGCTCCTGTCTGGACCCGAGCTAAGGC 692

1471 ATTATTTGATAAACACGTTGCCAGACTTG 1499
 691 TCTGTTCGACAAACACGTTCTGCGAATTG 663
```

FIGURE 6

(CONT.)

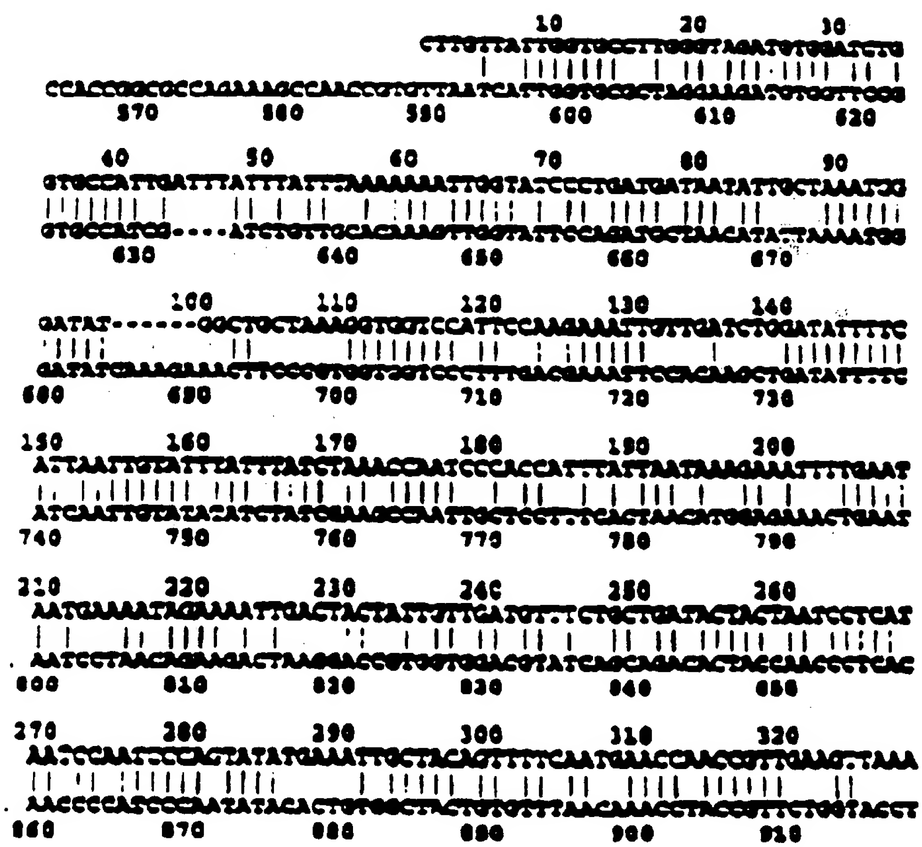


FIGURE 7

```
  1 MSKSPVILHLRAETKPLEARAALTPSTTKQLLDAGFEIYVEKESSQSTFDIKE 54
      ..|| |||||||||||| |.||||.||:.||||||||::||.|. ..|| .|
  1  MTAPVKLHLRAETKPLEHRSALTPTTTRKLLDAGFEVFVEKSPLRIFDDQE 52

 55  YEAVGAKIVPEGSWKTAPKERIIFGLKELPENETFPLIHEHIQFAHCYKD 103
     : .|||.:|.|||| .||.:|:|:||||||| |.||| |||||||||||||
 53  FVDVGATLVEEGSWVSAPEDRMIIGLKELPE.ESFPLSHEHIQFAHCYKD 101

104 QAGWQDVLKRFPQGNGILYDLEFLENDQGRRVAAFGFYAGFAGAAIGVLD 153
    |:||.|||.|||.|||.|||||||||:|.||||||||||.||||||||||| .
102 QGGWKDVLSRFPAGNGTLYDLEFLEDDNGRRVAAFGFHAGFAGAAIGVET 151

154 WSFKQLNGNTKGTKGEGEGGELPGVTPYPNENELIKDVKIELEKALTKNG 203
    |.|.|          ...::::||||.:||||.||:..:| :|. |:.| |
152 WAFQQ.........THPDSENLPGVSAYPNETELVDKIKKDLAAAVEK.G 191

204 GQYPKCLVIGALGRCGSGAIDLFKKIGIPDDNIAKWDMAETAKGGPFQEI 253
    :..|..|||||||||||||||| :|:||||::|| :|||.|| |||||||||
192 SKLPTVLVIGALGRCGSGAIDLARKVGIPEENIIRWDMNETKKGGPFQEI 241

254 VDLDIFINCIYLSKPIPPFINKEILNNENRKLTTIVDVSADTTNPHNPIP 303
    .| ||||||||||.|||||||| ::||.|.|||..|||||||||||||||:|
242 ADADIFINCIYLSQPIPPFINYDLLNKETRKLSVIVDVSADTTNPHNPVP 291

304 VYEIATVFNEPTVEVKLDKGPKLSVCSIDHLPSLLPREASEFFAKDLMPS 353
    ||.|||.|:.|||.|. . ||||||||||||||||||||||| |...|:||
292 VYTIATTFDHPTVPVETTAGPKLSVCSIDHLPSLLPREASEAFSEALLPS 341

354 LLELPNRDTSPVWVRAKQLFDKHVAR 379
    ||:||.|||.|||.|||.||||||| |
342 LLQLPQRDTAPVWTRAKALFDKHVLR 367
```

FIGURE 8

```
1   GGGATCCGCC CACGAGCACA TCCAGTTTGC CCACTGCTAC AAGCAACAGG
     vector
51  CCGGATGGAA TGACGTCCTC CGCCGATTCG CCCAGGGCAA GGGTACCCTC
101 TACGACCTCG AATTCCTCGA AGACCCCGTT TCCCACCGAC GTGTCGCCGC
151 ATTCGGTTTC CACGCCGGTT TCGCCGGCGC CGCCGCTGGT GCCCTCGCCT
201 TTGCCGCTCA GCAAACCCAA AATGGGCAAG GCAAGCTGGG CGAATTGAAG
251 CCGTACCCCA ATGAAGGCGA AATGGTCAAG GAAGTGAGTG AGGCGTTGGA
301 GGGCACCAAG GAAGGGAAGA AGGGAGTAAA GGTTTTGATC ATTGGAGCCT
351 TGGGACGATG TGGATCCGGT GCGGTTGACC TCTTCCGGAA GGCCGGCGTT
401 GCCGAGTACG TCTTTTTGTC CTCTCTCTCC CCTCTTGATC ATCTTGCTCA
          <-----------------------intron------------------
451 CGTCTTCTCG GCAAAATAGG GAAAATATCG TCAAGTGGGA TATGGCCGAG
    ------------------->
501 ACCGCCAAGG GCGGTCCCTT CCCCGAAATC CTGGACGTCG ACATTTTCAT
551 CAACTGCATG GGCTAGA
             vector
```

SEQUENCE OF NUCLEIC ACID AMPLIFIED FROM GENOMIC DNA OF <u>Cryptococcus neoformans</u>

FIGURE 9

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(74) Agent: HEAPHY, Barbara, A.; Banner & Allegretti, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).

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(54) Title: METHODS AND REAGENTS FOR DETECTING FUNGAL PATHOGENS IN A BIOLOGICAL SAMPLE

(57) Abstract

The present invention provides methods and materials for detecting the presence of a fungus in a biological sample. The inventive methods and materials exploit the fact that the amino acid sequence of the saccharopine dehydrogenase molecule expressed by Candida Albicans is highly conserved in fungi. Inventive hybridization probes, nucleic acids, PCR primers, antibodies, epitopes, reagents and methods are provided.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/16684

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07K16/14 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INFECTION AND IMMUNITY, vol. 62, no. 11, November 1994, WASHINGTON US, pages 5027-5031, XP002021822 GARRAD ET AL.: "Molecular and functional anylysis of the Lys1 gene of C. albicans" cited in the application see the whole document ---	1,2,4-7, 9-16,18, 19,21, 22,24, 25,27, 28, 30-35, 37,38, 40, 44-46, 51,52,57

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 1996	10.01.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Molina Galan, E

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	INFECTION AND IMMUNITY, vol. 60, no. 3, 1992, WASHINGTON US, pages 876-884, XP002021823 GOSHORN ET AL.: "Gene isolation by complementation in C. albicans and applications to mapping" cited in the application ---	
A	MOLECULAR AND CELLULAR BIOLOGY, vol. 10, no. 9, September 1990, WASHINGTON US, pages 4795-4806, XP002021824 XUAN ET AL.: "Overlapping reading frames at the LYS5 locus in the yeast Yarrowia lipolytica" cited in the application ---	
A	DATABASE MEDLINE A. N. 93054354, XP002021825 see abstract & JOURNAL OF BACTERIOLOGY, vol. 174, no. 22, November 1992, pages 7379-7384, GARRAD ET AL.: "Lysine biosynthesis in selected pathogenic fungi: characterization of lysine auxotrophs and the cloned LYS1 gene of Candida albicans" cited in the application ---	
A	WO,A,93 23568 (HOLMES ANN RACHEL ;CANNON RICHARD DAVID (NZ); JENKINSON HOWARD FRA) 25 November 1993 -----	

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 95/16684

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claims not searched: 3,8,17,20,23,26,29,36,39,41-43,47-50,53-56

No submision of valid sequence listing as requiered by Rule 5.2 PCT, only claims not containing seequences have been searched.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/16684

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9323568	25-11-93	AU-A- 4094293	13-12-93
		CA-A- 2136206	25-11-93
		EP-A- 0642588	15-03-95

Form PCT/ISA/210 (patent family annex) (July 1992)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 92 87 0160
Page 2

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,A	BIOTECHNOLOGY vol. 7, no. 3, March 1989, NEW YORK US pages 273 - 278 HOEKEMA, A., ET AL. 'The genetic engineering of two commercial potato cultivars for resistance to potato virus X' * the whole document *	5,9-14	
A	J. CELL. BIOCHEM. SUPPL. vol. 13D, 1989, page 346 YOUNG, M.J., ET AL. 'Barley yellow dwarf virus expression in wheat protoplasts and construction of synthetic genes to interfere with viral replication' * abstract M552 *	1-14	
A	EP-A-0 426 195 (ZAADUNIE) 8 May 1991 * page 10, line 25 - line 29; claims 1,2XIV-XIII *	1-14	TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	18 JANUARY 1993	MADDOX A.D.

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